CHARACTERIZATION AND COMPARISON OF PLANT AND CRUSTACEAN POLYPHENOL OXIDASES: KINETICS AND INHIBITION BY CHEMICAL METHODS

By

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS. ii

LIST OF TABLES. vii

LIST OF FIGURES viii

ABSTRACT. xii

INTRODUCTION. 1

LITERATURE REVIEW 4

Polyphenol Oxidase. 4
Enzymatic Browning (Melanosis). 6
Inhibition/Prevention of Melanosis. 9
Kojic Acid. 12
Supercritical Fluid (SCF) Treatment 12

CHARACTERIZATION AND COMPARISON OF CRUSTACEAN AND PLANT POLYPHENOL OXIDASES: KINETICS AND SOME PROPERTIES. 16

Introduction. 16
Materials and Methods 17
Extraction and Purification of Lobster Polyphenol Oxidase (PPO) 18
Extraction and Purification of Potato PPO 19
Extraction and Purification of Apple PPO. 20
Extraction and Purification of White Shrimp and Grass Prawn PPO 21
Protein Quantitation and Enzyme Purity Determination. 23
PPO Activity Determination. 23
pH Optima and Stability of Lobster PPO. 24
Activation Energy and Thermostability of Lobster PPO. 24
Molecular Weight Determination of Lobster PPO 25
Enzyme Kinetics Study 26
Results and Discussion. 26
Effect of pH on Lobster PPO Activity. 26
Effect of pH on Lobster PPO Stability 31
Effect of Temperature on Lobster PPO Activity and Stability . 31
Activation Energy (E_a) of Lobster PPO 39
Molecular Weight and Isoform Determination of Lobster PPO . . 40
Enzyme Kinetics of Lobster PPO. 43

Conclusion. 51

STRUCTURAL COMPARISON OF CRUSTACEAN, PLANT, AND MUSHROOM POLYPHENOL OXIDASES. 52

Introduction. 52
Materials and Methods 53
Extraction of Mushroom, Potato, Lobster, and Shrimp PPO . . . 53
Purification of Mushroom, Potato, Lobster, and Shrimp PPO . . 54
Protein Quantitation and Enzyme Purity Determination. 54
Enzyme Activity Assay 55
Anti-lobster PPO Antibody Production and Purification 55
Molecular Weight Determination of Anti-lobster PPO Antibody . 56
Antibody Titer Determination by Enzyme-linked Immunosorbent Assay (ELISA) 57
Analysis of Antigenic Properties of PPO 58
Immunoblotting. 58
Spectropolarimetric Analysis of PPO 59
Results and Discussion. 60
Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Profile of PPO 60
Antibody Production and Molecular Weight Determination. . . . 63
Antibody Titer Determination 63
Immunological Characteristics of PPO. 70
Spectropolarimetric Analysis of PPO 75
Conclusion. 89

INHIBITION MECHANISM OF KOJIC ACID ON SOME PLANT AND CRUSTACEAN POLYPHENOL OXIDASES 90

Introduction. 90
Materials and Methods 91
Extraction and Purification of Potato PPO 92
DEAE-cellulose Chromatography 93
Gel Filtration. 93
Extraction and Purification of Apple PPO. 94
Hydroxylapatite Chromatography. 94
Extraction and Purification of Grass Prawn PPO. 95
Extraction and Purification of Lobster PPO. 96
Extraction and Purification of white shrimp PPO 97
Protein Quantitation and Enzyme Purity Determination. 98
Enzyme Activity Assay 98
Effect of Kojic Acid on Enzyme Activity 99
Enzyme Kinetics Study 100
Effect of Pre-incubation Temperature on PPO Inhibition by Kojic Acid. 102
Effect of Kojic Acid on the Hydroxylation Capability of PPO . 103
Effect of Kojic Acid on Oxygen Uptake by PPO Reaction 103
Effect of Kojic Acid on Reduction of Cupric Copper. 104
Effect of Kojic Acid on Quinone Products. 104
Enzymatic Activities of Kojic Acid-treated PPO. 105
Statistical Analysis. 106

Results and Discussion. 107
Effect of Kojic Acid on Mushroom PPO Activity 107
Effect of Kojic Acid on Potato PPO Activity 107
Effect of Kojic Acid on Apple PPO Activity. 114
Effect of Kojic acid on Crustacean PPO Activity 117
Enzyme Kinetics 117
Effect of Pre-incubation Temperature on PPO Inhibition by Kojic Acid. 129
Effect of Kojic Acid on the Hydroxylation Capability of PPO . 129
Effect of Kojic Acid on Oxygen Uptake by PPO Reaction 131
Effect of Kojic Acid on Reduction of Cupric Copper. 131
Effect of Kojic Acid on Quinone Products. 135
Enzymatic Activities of Kojic Acid-treated PPO. 140
Conclusion. 140

EFFECT OF CARBON DIOXIDE ON THE INACTIVATION OF PLANT AND CRUSTACEAN POLYPHENOL OXIDASES. 143

Introduction. 143
Materials and Methods 144
Extraction and Purification of Lobster, Shrimp, and Potato PPO 144
Protein Quantitation and Enzyme Purity Determination. 145
Enzyme Activity Assay 146
Effect of Carbon Dioxide (1 atm) on PPO Activity. 146
pH Control Study. 147
Effect of High Pressure (58 atm) Carbon Dioxide on PPO Activity. 147
Kinetics of PPO Inactivation. 150
Nondenaturing Polyacrylamide Gel Electrophoresis of Carbon Dioxide (1 atm)-treated PPO 151
Mass Balance of High Pressure Carbon Dioxide-treated and Nontreated PPO. 151
Polyacrylamide Gel Isoelectric Focusing of Carbon Dioxide-treated PPO 152
Spectropolarimetric Analysis of PPO 153
Study of Restoration of Carbon Dioxide-treated PPO Activity . 153
Results and Discussion. 154
Effect of Carbon Dioxide (1 atm) on PPO Activity. 154
Effect of Nitrogen on PPO Activity. 157
Effect of High Pressure Carbon Dioxide on PPO Activity. . . . 159
Kinetics of PPO Inactivation. 166
Polyacrylamide Gel Electrophoresis of Carbon Dioxide (1 atm)-treated PPO 168
Mass Balance of High Pressure Carbon Dioxide-treated and Nontreated PPO 168
Polyacrylamide Gel Isoelectric Focusing of Carbon Dioxide-treated PPO 172
Spectropolarimetric Analysis of High Pressure Carbon Dioxide-treated PPO 172
Restorative Ability of Carbon Dioxide-treated PPO Activity. . 186
Conclusion. 189

CONCLUSIONS 197

REFERENCE LIST. 199

BIOGRAPHICAL SKETCH 216

LIST OF TABLES

TABLE		PAGE
1	Comparison of Kinetic Properties of Polyphenol Oxidase (PPO) from Various Sources	30
2	Comparison of Polyphenol Oxidase (PPO) Activity between Florida Spiny Lobster and Western Australian Lobster	49
3	Secondary Structure Estimates of Various Polyphenol Oxidases (PPOs) from Far UV Circular Dichroic Spectra	88
4	Inhibitory Mechanism of Kojic Acid on Polyphenol Oxidase Obtained from Various Sources.	124
5	Effect of Different Pre-incubation Temperatures on the Inhibition of Various Polyphenol Oxidases (PPOs) by Kojic Acid	130
6	Inhibitory Effect of Kojic Acid on the Consumption of Oxygen by Polyphenol Oxidase.	134
7	Enzymatic Activity of Kojic Acid-treated Polyphenol Oxidase Following Kojic Acid Removal	141
8	Effect of Nitrogen on Florida Spiny Lobster Polyphenol Oxidase Activity	158
9	Kinetic Parameters of Florida Spiny Lobster Polyphenol Oxidase Inactivation by Carbon Dioxide (1 atm) at Various Temperatures	167
10	Mass Balance of Protein Contents from High Pressure CO_2-treated and Nontreated PPO	171
11	Secondary Structure Estimates of Nontreated Control and High Pressure CO_2-treated Florida Spiny Lobster, Brown Shrimp, and Potato Polyphenol Oxidases (PPOs) from Far UV Circular Dichroic Spectra	185

LIST OF FIGURES

FIGURE		PAGE
1	Mechanism of Polyphenol Oxidase-catalyzed Reaction	8
2	Structure of Kojic Acid (5-hydroxy-2-hydroxymethyl-γ-pyrone).	14
3	Effect of pH on the Activity of Polyphenol Oxidase (PPO) Isolated from Florida Spiny Lobster and Western Australian Lobster	28
4	Effect of pH on the Stability of Polyphenol Oxidase (PPO) Isolated from Florida Spiny Lobster and Western Australian Lobster	33
5	Effect of Temperature on the Activity of Polyphenol Oxidase (PPO) Isolated from Florida Spiny Lobster and Western Australian Lobster	35
6	Effect of Temperature on the Stability of Polyphenol Oxidase (PPO) Isolated from Florida Spiny Lobster and Western Australian Lobster	38
7	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE, 7.5% Gel) Profile of Polyphenol Oxidase from Florida Spiny Lobster (FSL) and Western Australian Lobster (WAL).	42
8	Double Reciprocal Plots for the Oxidation of DL-DOPA and Catechol by Florida Spiny Lobster Polyphenol Oxidase (PPO).	45
9	Double Reciprocal Plots for the Oxidation of DL-DOPA and Catechol by Western Australian Lobster Polyphenol Oxidase (PPO).	47
10	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE, 7.5% Gel) Profile of Polyphenol Oxidase from Mushroom, Potato, Florida Spiny Lobster, White Shrimp, and Brown Shrimp	62
11	Profile of Anti-lobster PPO Antibody Production in Immunized Hen during the Immunization Period	65

12 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE, 7.5% Gel) Profile of Anti-lobster PPO Antibody 67

13 The Titer Determination of Antibody against Florida Spiny Lobster PPO versus Mushroom, Potato, Florida Spiny Lobster, White Shrimp, and Brown Shrimp PPO. 69

14 Analysis of Antigenic Properties of Purified Mushroom, Potato, Florida Spiny Lobster, White Shrimp, and Brown Shrimp PPO by Competitive ELISA. 72

15 Profile of Protein Standard (SDS-6H), Purified Mushroom, Potato, Florida Spiny Lobster, White Shrimp, and Brown Shrimp PPO, and Crude Lobster PPO Preparation on a Nitrocellulose Membrane. 74

16 Determination of the Specific Reactivity of Anti-lobster PPO Antibody with Crude Lobster PPO Preparation, Purified Mushroom, Potato, Florida Spiny Lobster, White Shrimp, and Brown Shrimp PPO as well as SDS-6H Protein Standard as Analyzed by Immunoblotting Technique 77

17 The far UV Circular Dichroic Spectra of Mushroom PPO . . . 79

18 The far UV Circular Dichroic Spectra of Potato PPO 81

19 The far UV Circular Dichroic Spectra of Florida Spiny Lobster PPO. 83

20 The far UV Circular Dichroic Spectra of White Shrimp PPO . 85

21 The far UV Circular Dichroic Spectra of Brown Shrimp PPO . 87

22 Effect of Concentration-related Inhibitory Effect of Kojic Acid on Mushroom Tyrosinase (PPO) Activity on DL-β-3,4-dihydroxyphenylalanine (DL-DOPA) 109

23 The Concentration-related Inhibitory Effect of Kojic Acid on the Oxidation of DL-DOPA and L-Tyrosine by Mushroom PPO. 111

24 The Concentration-related Inhibitory Effect of Kojic Acid on the Oxidation of Chlorogenic Acid and Catechol by Potato PPO 113

25 The Concentration-related Inhibitory Effect of Kojic Acid on the Oxidation of 4-Methylcatechol and Chlorogenic Acid by Apple PPO. 116

26 The Concentration-related Inhibitory Effect of Kojic Acid on the Oxidation of L-DOPA and Catechol by White Shrimp PPO. 119

27 The Concentration-related Inhibitory Effect of Kojic Acid on the Oxidation of DL-DOPA and Catechol by Grass Prawn PPO. 121

28 The Concentration-related Inhibitory Effect of Kojic Acid on the Oxidation of DL-DOPA and Catechol by Lobster PPO. 123

29 Effect of Kojic Acid on the Hydroxylation of Monohydroxyphenol by Mushroom Tyrosinase (PPO). 133

30 Effect of Kojic Acid on Reduction of Cu^{2+} to Cu^{+} in a Model System 137

31 Change in Absorption Spectra of Dopaquinone due to the Addition of Kojic Acid. (a) Dopaquinone Produced from the Action of Mushroom PPO on DL-DOPA; and (b) Dopaquinone Plus Kojic Acid. 139

32 Apparatus Used for Studying Polyphenol Oxidase (PPO) Inactivation by High Pressure CO_2 149

33 Effect of Carbon Dioxide (1 atm) on the Change in pH and Enzyme Activity of Florida Spiny Lobster PPO Heated at 33° (a), 38° (b), or 43°C (c). 156

34 Effect of High Pressure (58 atm) Carbon Dioxide on the Change in pH and Enzyme Activity of Florida Spiny Lobster PPO heated at 43°C 161

35 Effect of High Pressure (58 atm) Carbon Dioxide on the Change in pH and Enzyme Activity of Brown Shrimp PPO heated at 43°C 163

36 Effect of High Pressure (58 atm) Carbon Dioxide on the Change in pH and Enzyme Activity of Potato PPO heated at 43°C 165

37 Nondenaturing Polyacrylamide Gel Electrophoresis (PAGE, 7.5% Gel) Profile of Carbon Dioxide (1 atm)-treated Florida Spiny Lobster PPO. 170

38 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE, 7.5% Gel) Profile of High Pressure (58 atm) Carbon Dioxide-treated Florida Spiny Lobster PPO 174

39 Polyacrylamide Gel Isoelectric Focusing (IEF, 5% Gel) Profile of Carbon Dioxide (1 atm)-treated Florida Spiny Lobster PPO. 176

40 Polyacrylamide Gel Isoelectric Focusing (IEF, 5% Gel) Profile of High Pressure (58 atm) Carbon Dioxide-treated Florida Spiny Lobster PPO. 178

41 Comparison of Far UV Circular Dichroic Spectra of Nontreated Control and High Pressure (58 atm) Carbon Dioxide-treated Florida Spiny Lobster PPO. 180

42 Comparison of Far UV Circular Dichroic Spectra of Nontreated Control and High Pressure (58 atm) Carbon Dioxide-treated Brown Shrimp PPO 182

43 Comparison of Far UV Circular Dichroic Spectra of Nontreated Control and High Pressure (58 atm) Carbon Dioxide-treated Potato PPO 184

44 The Restorative Ability of Carbon Dioxide (1 atm)-treated Florida Spiny Lobster PPO Activity and the Pertinent Environmental pH Changes during Frozen-storage 188

45 The Restorative Ability of High Pressure (58 atm) Carbon Dioxide-treated Florida Spiny Lobster PPO Activity and the Pertinent Environmental pH Changes during Frozen-storage. 191

46 The Restorative Ability of High Pressure (58 atm) Carbon Dioxide-treated Brown Shrimp PPO Activity and the Pertinent Environmental pH Changes during Frozen-storage 193

47 The Restorative Ability of High Pressure (58 atm) Carbon Dioxide-treated Potato PPO Activity and the Pertinent Environmental pH Changes during Frozen-storage 195

Abstract of Dissertation Presented to the Graduate School
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CHARACTERIZATION AND COMPARISON OF PLANT AND CRUSTACEAN POLYPHENOL OXIDASES: KINETICS AND INHIBITION BY CHEMICAL METHODS

By

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Chairman: M.R. Marshall
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The characterization and comparison of polyphenol oxidase (PPO, E.C. 1.14.18.1) from crustaceans (lobster and shrimp) and plants were made. PPO from various plant and crustacean sources varied with respect to molecular weight, pH and temperature effects on activity and stability, substrate specificity, and other kinetic parameters. Differences in conformational structure among PPOs from mushroom, potato, lobster, white shrimp, and brown shrimp were investigated using spectropolarimetric and immunological methods. Varied secondary structures were observed among these PPOs, although they were shown to possess similar antigenic determinants.

Kojic acid [5-hydroxy-2-(hydroxymethyl)-γ-pyrone], a fungal metabolite, exhibited competitive and/or mixed types of inhibition on the oxidation of various phenolic substrates (L-tyrosine, DL-β-3,4-dihydroxy phenylalanine, 4-methylcatechol, catechol, and chlorogenic acid) by mushroom, potato, apple, and crustacean (lobster, grass prawn, and white

shrimp) PPOs. In addition, kojic acid was capable of reducing *o*-quinones back to diphenols and prevented melanin formation.

The inactivation of lobster, brown shrimp, and potato PPO by CO_2 was studied. When exposed to CO_2 (1 atm) at 33°, 38°, or 43°C, lobster PPO showed a decline in enzyme activity with treatment time. Studies on inactivation kinetics revealed that PPO was more labile to CO_2 and heat than to heat alone. The use of polyacrylamide gel electrophoresis showed that there were no differences in protein patterns and isoelectric profiles between the CO_2 (1 atm)-treated and untreated PPO. When lobster, brown shrimp, and potato PPOs were subjected to high pressure (58 atm) CO_2 at 43°C, the inactivation of these PPOs followed trends similar to the atmospheric CO_2 experiments. Crustacean PPOs, however, were more susceptible to inactivation by high pressure CO_2 than by atmospheric CO_2. Differences in the secondary structures between the high pressure CO_2-treated and the nontreated PPO were evident by spectropolarimetric analysis.

INTRODUCTION

Undesirable enzymatic browning causing the discoloration or formation of black spots (melanosis) by polyphenol oxidase (E.C. 1.14.18.1; PPO) on the surface of many vegetable, fruit, and seafood (crustacean) products has been of great concern to food scientists. For food processors, the formation of melanin pigments not only imparts the problems in sensory attributes and, hence, the marketability of the product, but often lower its nutritive value as well (Synge, 1975).

Kinetic properties have been studied for PPO from various sources of vegetables and fruits (Schwimmer, 1981) and more recently from crustaceans (Ferrer et al., 1989a; Rolle et al., 1991; Simpson et al., 1987, 1988a). However, little comparative biochemical information exists between PPO from plants and crustaceans. A preliminary study conducted in this laboratory revealed that Western Australian lobster (*Panulirus cygnus*) was far less susceptible to melanosis during storage at refrigeration temperature than Florida spiny lobster (*Panulirus argus*) maintained under the same conditions. It is speculated that the susceptibility to melanosis could be attributed to the difference in PPO activity between these two lobster species. Thus, the first objective of this study was to characterize and determine the kinetic properties of PPO enzymes from these two lobsters as well as from other crustacean and plant sources.

Many chemical and physical methods have been studied for their effectiveness on the inhibition of enzymatic browning. Browning may be

prevented not only by inactivating the enzyme, but also by eliminating substrate (O_2 or polyphenol) necessary for the reaction, or by reacting with the products of the enzyme reaction thus preventing the formation of the quinones necessary for the preceding non-enzymatic steps. Although some chemicals have been shown to inhibit PPO activities, their use in food processing is restricted by many concerns such as toxicity, wholesomeness, effect on taste, flavor, texture, etc. (Vamos-Vigyazo, 1981). Kojic acid (5-hydroxy-2-hydroxymethyl-γ-pyrone), a fungal metabolite produced by many species of *Aspergillus* and *Penicillium* (Kinosita and Shikata, 1964), has been reported for its inhibitory effect on mushroom PPO (Saruno et al., 1979). Kojic acid mixed with ascorbic acid and citric acid constitutes a Japanese commercial product which is used as a tyrosinase inhibitor in foods. Since only limited information was available on the inhibitory activity and mechanism of kojic acid on PPO, the second objective of this study was to investigate the inhibitory activity of this compound on crustacean (Florida spiny lobster, white shrimp, and grass prawn) mushroom, and plant (potato and apple) PPO and to elaborate the mechanisms of inhibition.

In past years, treatment in an atmosphere modified with carbon dioxide has been used as an application for retarding enzyme activity, to preserve food quality and to extend the shelf-life of food products. Recently, inactivation of peroxidase, PPO, pectinesterase, α-amylase, glucose oxidase, lipase, or catalase by supercritical fluid using CO_2 as the solvent has been reported by many workers (Arreola, 1990; Christianson et al., 1984; Taniguchi et al., 1987; Zemel, 1989). However, information concerning the inhibitory effect and the inhibition mechanism of CO_2 on

plant and crustacean PPO was limited. Thus, the third objective of this study was to investigate the effect of CO_2 (atmospheric and high pressure) on the inhibition of lobster, brown shrimp, and potato PPO.

LITERATURE REVIEW

Polyphenol Oxidase

Polyphenol oxidase (PPO) (E.C. 1.14.18.1.), also known as tyrosinase, polyphenolase, phenolase, catechol oxidase, cresolase, and catecholase, is widely distributed in nature (Schwimmer, 1981). In addition to its general occurrence in plants, it can also be found in microorganisms, especially fungi, and in some animal tissue (Brown, 1967). PPO plays an important role in the resistance of plants to microbial or viral infections and probably to adverse climate (Vamos-Vigyazo, 1981). The vast literature dealing with the role of the PPO-polyphenol system in plant pathology has been extensively reviewed (Bonner, 1957; Farkas and Kiraly, 1962). A simplified explanation of this role in the resistance of plants to infections is that the quinones formed upon the action of the enzyme undergo secondary polymerization reactions yielding dark, insoluble polymers; the tissues impregnated with these polymers act as barriers to prevent further spreading of the infection. This is considered by some authors to be the main function of the enzyme (Macrae and Duggleby, 1968). However, plants resistant to adverse climatic conditions have, in general, higher PPO activities than susceptible varieties (Khrushcheva and Krehin, 1965).

In addition to the involvement in phenolic compounds biosynthesis, the PPO enzymes also indirectly participate in auxin biosynthesis. The

primary products of its action on *o*-diphenols, the *o*-quinones, react with tryptophan to form indole acetic acid via indolepyruvic acid (Gordon and Paleg, 1961). Thus, PPO together with auxin degrading enzyme (peroxidase), might play a role in plant growth regulation (Vamos-Vigyazo, 1981). The quinones formed upon PPO action may also participate in reactions similar to those leading to nonenzymatic browning and humification and thus contribute to producing organic matter of soil (Synge, 1975).

PPO from insects and crustaceans plays an important role in the sclerotization during molting (Andersen, 1971; Brunet, 1980; Cobb, 1977; Ferrer et al., 1989a; Summers, 1967; Vinayakam and Nellaiappan, 1987). In this process, PPO oxidizes diphenols to quinones, which interact with certain side groups on adjacent proteins, thus linking them together (Stevenson, 1985). N-Acetyldopamine (Andersen, 1979) and 3,4-dihydroxybenzoic acid (Pryor et al., 1962) have been identified as the cross-linking agents in crustacean and cockroach, respectively. Another function closely related to plant systems was PPO's involvement in the process of wound repair and calcification of the cuticle (Stevenson, 1985).

Most PPO's are copper-containing enzymes which catalyze two entirely different reactions: (a) the hydroxylation of monophenols to the corresponding *o*-dihydroxy compounds, which is called cresolase activity, and (b) the oxidation of *o*-dihydroxy phenols to *o*-quinones, which is termed catechol activity. The activity of cresolase involves three steps which can be represented by the following equation (Mason, 1956):

$$\text{Protein-Cu}^{2+}\text{-O}_2 + \text{monophenol} \rightarrow \text{Protein-Cu}^{2+} + o\text{-quinone} + \text{H}_2\text{O}$$

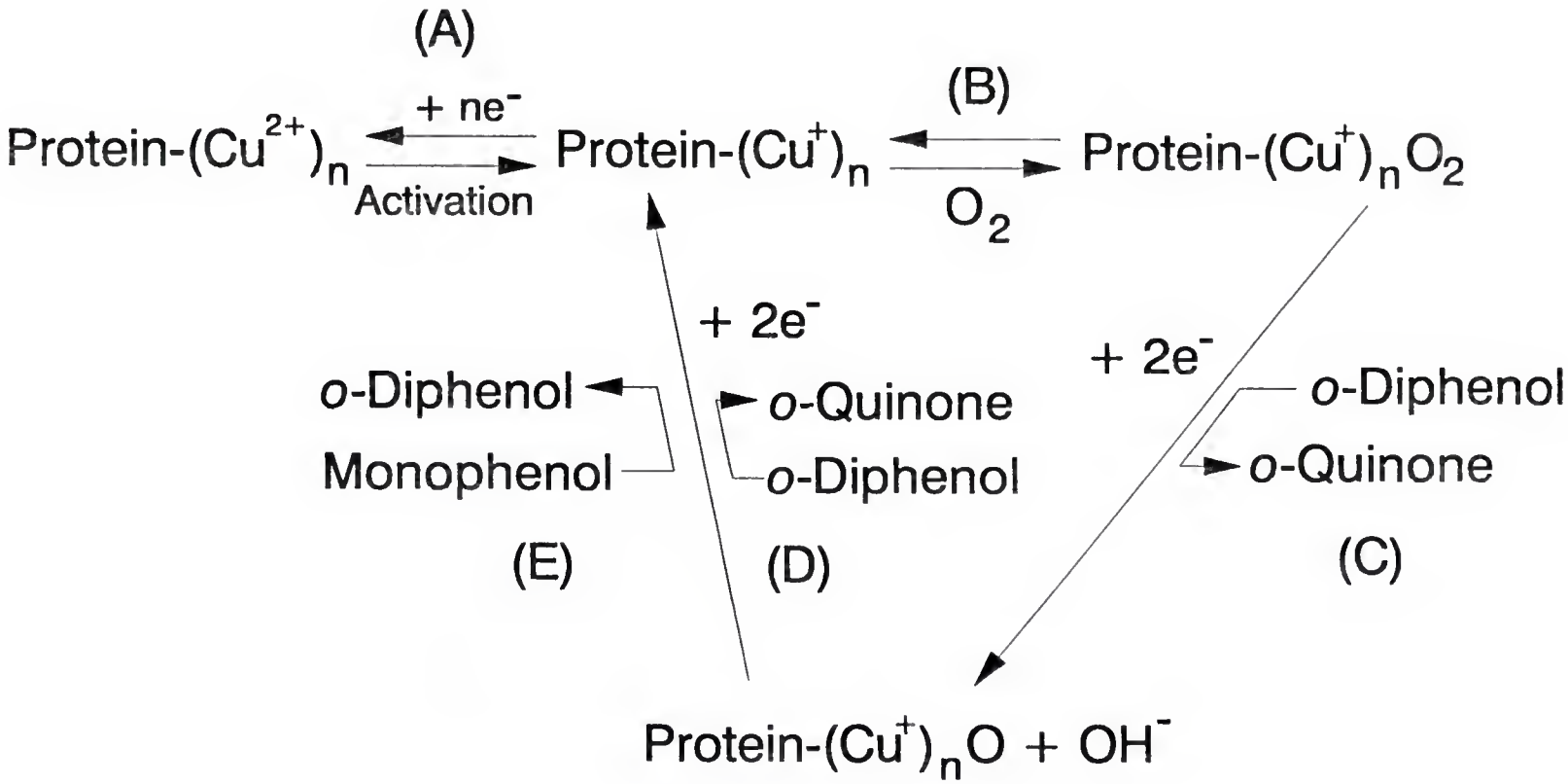
The protein-copper-oxygen complex is formed by combining one molecule of oxygen with the protein to which two adjacent cuprous atoms are attached. Catecholase activity involves the oxidation of 2 molecules of *o*-diphenols to 2 molecules of *o*-quinones, resulting in the reduction of one molecule of oxygen to two molecules of water. The sequence for the PPO-catalyzed reaction proposed by Mason (1957) is shown in Figure 1. The enzyme-oxygen complex serves as the hydroxylating or dehydroxylating intermediate, and $(Cu)_n$ represents the actual charge designation of the copper at the active site. The overall reaction involves the use of one molecule of oxygen; one atom of which goes into the formation of diphenol, and the other is reduced to water. Substrate specificity varies considerably for PPO from various sources (Aurand and Woods, 1973).

Enzymatic Browning (Melanosis)

Food scientists' primary interest in PPO is in the enzymatic browning phenomenon and its effect on food quality. *o*-Quinones, the primary products of PPO oxidative reaction can (a) interact with each other to form high molecular weight polymers, (b) form macromolecular complexes with amino acids or proteins, and (c) oxidize compounds of lower oxidation-reduction potentials (Mathew and Parpia, 1971). Enzymatic browning is a desirable process for the manufacture of black tea, sultana grape, and ground coffee bean (De Amorim and Silva, 1968; Grncarevic and Hawker, 1971; Takeo, 1966).

Unfavorable darkening on the surface of many fruits, vegetables, and seafood products is primarily due to the indirect consequence of PPO oxidizing phenols to orthoquinones, which in turn rapidly polymerize to

Figure 1. Mechanism of Polyphenol Oxidase-catalyzed Reaction: Activation of Polyphenol Oxidase (A), Two-step Four-Electron Reduction of Oxycupropolyphenoloxidase (B-C-D), and the Associated Hydroxylation of Monophenols (B-C-E)
Source: Mason, 1957


(A)
+ ne⁻
Protein-(Cu²⁺)n
Activation
Protein-(Cu⁺)n
(B)
Protein-(Cu⁺)nO2
O2
+ 2e⁻
o-Diphenol
Monophenol
o-Quinone
o-Diphenol
(E)
(D)
+ 2e⁻
o-Diphenol
o-Quinone
(C)
Protein-(Cu⁺)nO + OH⁻

form brown pigments or melanins (Joslyn and Ponting, 1951). Enzymatic browning of fruits, vegetables, and seafood products (mainly shrimp and lobster) due to PPO activity has been extensively studied (Ferrer et al., 1989a; Flurkey and Jen, 1978; Harel et al., 1966; Koburger et al., 1985; Macrae and Duggleby, 1968; Madero and Finne, 1982; Mapson et al.,1963; Mosel and Hermann, 1974; Palmer, 1963; Patil and Zucker, 1965; Savagaon and Sreenivasan, 1978; Sciancalepore and Longone, 1984; Simpson et al., 1987, 1988a; Walker, 1962, 1964; Weurman and Swain, 1955). Although not harmful to consumers, it is the formation of melanins on the surface of these products by PPO that decreases their market value, making them unappealing and the perception of spoilage (Alford and Fieger, 1952; Bailey and Fieger, 1954; Bailey et al., 1960a, 1960b; Faulkner et al., 1954; Ogawa et al., 1984).

Inhibition/Prevention of Melanosis

Undesirable enzymatic browning occurring in agricultural products causes an enormous economical loss for food industries. Thus, in order to reduce this loss, the occurrence of melanosis on food products must be prevented or inhibited. Inhibition of PPO and prevention of enzymatic browning are often treated as one and the same subject. Browning can be prevented by inactivating PPO, by eliminating one of the substrates necessary for the reaction (O_2 and the polyphenols), or by reacting the products of PPO metabolism which in turn inhibit the formation of the colored compounds produced in the secondary, non-enzymatic reaction steps. As PPO is a metalloprotein with copper as the prosthetic group (Bailey et al., 1960a; Bendall and Gregory, 1966; Mayer, 1962; Smith and Kruger,

1962), the activity of this enzyme can be inhibited by metal-chelating agents. The effects of cyanide, carbon monoxide, sodium-diethyl-dithiocarbamate (DIECA), mercaptobenzothiazole, dimercaptopropanol, potassium methyl xanthate, fluoride, azide, borate, benzoic acid, small peptides, polyvinylpyrrolidone (PVP), 2,3-naphthalenediol, ascorbic acid, and dichlorodifluoromethane on plant PPO activity have been extensively studied; these chemical inhibitors act primarily on the enzyme (Bedrosian et al., 1959; Harel et al., 1967; Jones et al., 1965; Mayer et al., 1964; Palmer and Roberts, 1967; Pierpoint, 1966; Pifferi et al., 1974; Robb et al., 1966; Taeufel and Voigt, 1964; Walker, 1964, 1975, 1976; Walker and Wilson, 1975; Yasunobu and Norris, 1957).

Other compounds acting as reducing agents which reduce *o*-quinones to diphenols or react with plant PPO substrates to lessen the extent of enzymatic browning are ascorbic acid, SO_2, sodium sulfite, sodium bisulfite, sodium metabisulfite, potassium metabisulfite, cysteine, 2-mercaptoethanol, glutathione, benzenesulphinic acid, DIECA, Na-ethyl xanthate, and PVP (Cash et al., 1976; Embs and Markakis, 1965; Feinberg et al., 1976; Haisman, 1974; Hope, 1961; Markakis and Embs, 1966; Mapson, 1965; Mapson and Wager, 1961; Muneta, 1966; Muneta and Walradt, 1968; Muneta and Wang, 1977; Ponting, 1960; Ponting and Jackson, 1972; Sayavedra-Soto and Montgomery, 1986; Singh and Ahlawati, 1974; Taeufel and Voigt, 1964; Vamos-Vigyazo, 1981; Walker, 1964). For the prevention of melanosis in crustaceans, numerous agents including ascorbic acid and sodium ascorbate, ascorbate and citrate, L-tyrosine, L-methionine, citric acid, L-cysteine, sodium diethyl dithiocarbamate, sodium tripolyphosphate (STP), sodium sulfite, and sodium bisulfite have been broadly studied

(Antony and Nair, 1975; Bailey and Fieger, 1954; Faulkner et al., 1954; Ferrer et al., 1989b; Fink, 1988; Madero, 1982; Madero and Finne, 1982).

Many methods are available for inhibiting/inactivating PPO. The application of these methods or chemicals for inhibition in the food industry, however, is limited. Problems related to off-flavor, off-odor, toxicity, and economic feasibility all affect the application of chemicals (Eskin et al., 1971). Sulfiting agents listed as Generally Recognized As Safe (GRAS) by the Food and Drug Administration (FDA) since 1959 (FDA, 1984) have been widely used to prevent melanosis of agricultural and seafood products. However, due to the recent health concern and questions regarding the safety of sulfiting agents, the FDA currently proposed revoking the GRAS status of these additives for use on fruits and vegetables intended to be served or sold fresh or raw to consumers. The FDA also requires label declaration of these agents added to food or as an ingredient whenever they are present in the finished product at a level equal to or higher than 10 ppm as SO_2 (FDA, 1985). Furthermore, the FDA requires that sulfite-treated shrimp products having residue levels greater than 10 ppm must bear a label stating the presence of these additives (FDA, 1985).

The maximum allowable residual level of sulfite on shrimp in the raw edible portion is 100 ppm as SO_2 (Marshall et al., 1984), which is not hazardous to the majority of the population. After exposure to these additives even at this level, however, people still suffer adverse reactions ranging from mild to severe symptoms (Lecos, 1986). Therefore, the search for an alternative which can inhibit melanosis but does not cause adverse reactions becomes a necessity.

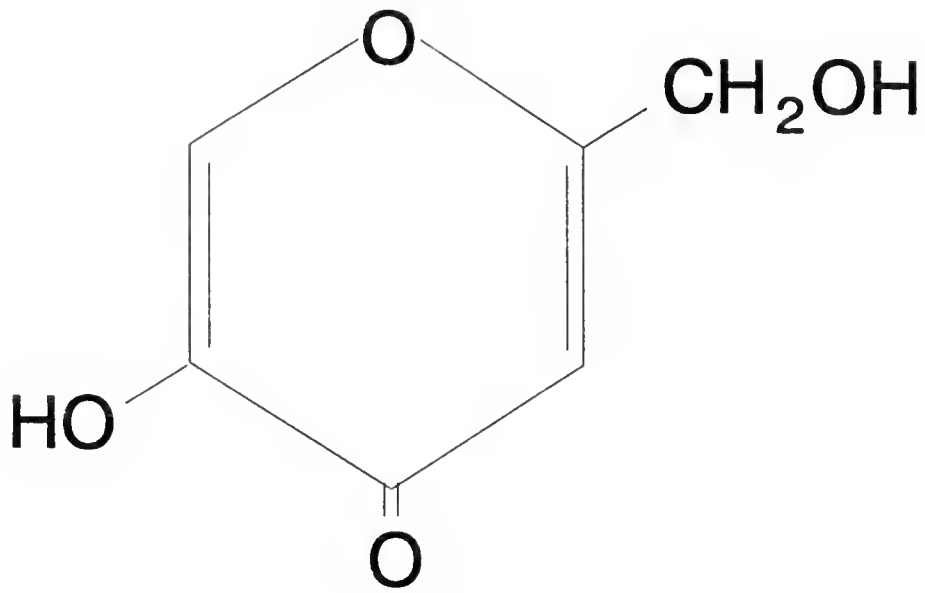
Kojic Acid

The inhibitory effect of some tyrosine-3-hydroxylase inhibitors of microbial origins such as aquayamycin, chrothiomycin, and oudenone on PPO activity has been studied (Ayukawa et al., 1968, 1969; Nagatsu et al., 1968; Sezaki et al., 1968; Umezawa et al., 1970). Kojic acid (5-hydroxy-2-hydroxymethyl-γ-pyrone) (Figure 2) produced in the culture filtrate of *Aspergillus albus* was shown *in vitro* to inhibit the activity of mushroom tyrosinase using DOPA and tyrosine as substrates (Saruno et al., 1979). At low concentration, kojic acid was found to inhibit the *in vitro* oxidation of a number of D-amino acids, L-phenylalanine and a few related compounds (Bajpai et al., 1981). Production of kojic acid, generally considered as a secondary metabolite from carbohydrate metabolism under aerobic conditions, has been extensively studied (Bajpai et al., 1981, 1982a, 1982b; Morton et al., 1945; Saruno et al., 1979). Although kojic acid can be considered a potential candidate to inhibit melanosis, the use of this chemical, depends not only on its production but also its potential toxicity to humans.

Supercritical fluid (SCF) Treatment

Intensive study of supercritical fluids for extraction of food components has mostly concentrated on decaffeination, deodorization, aroma recovery, oil recovery, oil refining, and fractionation (Bulley et al., 1984; Christianson et al., 1984; Friedrich and Pryde, 1984; Lee et al., 1986; Maddocks and Gibson, 1977; Peter and Brunner, 1978; Snyder et al., 1984; Schultz et al., 1967; Stahl et al., 1978, 1980, 1984; Taniguchi et al., 1985; Vollbrecht, 1982; Zosel, 1979, 1981). Modified atmospheres

Figure 2. Structure of Kojic Acid (5-hydroxy-2-hydroxymethyl-γ-pyrone)



Kojic acid

(5-hydroxy-2-hydroxymethyl-gamma-pyrone)

(carbon dioxide or CO_2-saturated brines) have been used successfully to preserve food quality and extend shelf-life (Barnett et al., 1978; Brown et al., 1980; Bullard and Collins, 1978; Gee and Brown, 1987a, 1987b; Lannelongue et al., 1982; Longard and Reiger, 1974; Shewan, 1950; Vernath and Robe, 1979; Villemure et al., 1986; Woyewoda et al., 1984; Yokoseki et al., 1956). Studies of seafood preservation using a modified atmosphere of CO_2 showed that the lower internal pH of the tissue was due to exposure of the external CO_2 present and resulted in a rapid acidification of the internal cellular environment (Aickin and Thomas, 1975; Boron and DeWeer, 1976; Thomas, 1974; Thomas and Ellis, 1976; Turin and Warner, 1977). Intracellular and extracellular acidification of tissue could produce an antimicrobial effect and also influence many different enzymatic activities (Parkin et al., 1981). SCF possesses physicochemical properties intermediate between those of liquids and gases which enhance its efficacy as solvents; the higher gas density gives good solvent power, while the lower viscosity and higher diffusivity provide the SCF with higher gas permeability (Rizvi et al., 1986). Carbon dioxide is used as a solvent for SCF because it is nontoxic, nonflammable, inexpensive and readily available (Hardardottir and Kinsella, 1988) and it has a relatively low critical temperature (33.1°C) and pressure (72 atm) (Rizvi et al., 1986). Eldridge et al. (1986) reported that SCF produced minimal detrimental effects on the functional properties of proteins. The application of SCF using CO_2 as a solvent in seafood processing could be a benefit because it possesses characteristics of antimicrobial action, lipid (cholesterol) reduction, and enzymatic inhibitory activities.

CHARACTERIZATION AND COMPARISON OF CRUSTACEAN AND PLANT POLYPHENOL OXIDASES: KINETICS AND SOME PROPERTIES

Introduction

Polyphenol oxidase (PPO) (E.C. 1.14.18.1.), also known as tyrosinase, polyphenolase, phenolase, catechol oxidase, cresolase, and catecholase, is widely distributed in nature (Schwimmer, 1981). In addition to its general occurrence in plants, it can also be found in microorganisms, especially in fungi, and in some animal organs (Brown, 1967). Unfavorable darkening of many fruits and vegetables after cutting is primarily due to the action of this enzyme. Enzymatic browning of fruits and vegetables due to PPO activity has been widely reported (Flurkey and Jen, 1978; Harel et al., 1966; Macrae and Duggleby, 1968; Mapson et al., 1963; Mosel and Herrman, 1974; Palmer, 1963; Patil and Zucker, 1965; Sciancalepore and Longone, 1984; Walker, 1962, 1964; Weurman and Swain, 1955). However, the role of PPO in crustaceans is not well documented considering the variation in susceptibility to melanosis.

PPO plays an important role in the sclerotization of insects and crustaceans during the molting cycle (Andersen, 1971; Brunet, 1980; Summers, 1967; Vinayakam and Nellaiappan, 1987). However, it is the formation of melanins causing darkening on the surface of seafood products due to PPO action which is of concern to the seafood industry. PPO enzymes from various crustaceans have been partially characterized (Madero and Finne, 1982; Nakagawa and Nagayama, 1981; Simpson et al., 1987,

1988a). Although kinetic properties have been studied for PPO from various sources of vegetables and fruits (Schwimmer, 1981) and more recently from crustaceans (Chen et al., 1991a; Ferrer et al., 1989a; Rolle et al., 1991), little comparative biochemical information exists between PPO from plants and crustaceans. A preliminary study conducted in this laboratory revealed that Western Australian lobsters were far less susceptible than the Florida spiny lobsters to melanosis during storage at refrigeration temperature. A similar phenomenon was observed on pink shrimp (*Penaeus duorarum*) in comparison to white shrimp (*Penaeus setiferus*) (Simpson et al., 1988a). These workers characterized the PPO properties and they attributed the difference in susceptibility to melanosis to the varied physiological efficiency of the enzyme and the distribution of phenolic substrates in the shrimp (Simpson et al., 1988a). Koburger et al. (1985) also showed that South African lobster tails were less susceptible to melanosis than Florida spiny lobster tails. Since the water habitat of Western Australian lobster is colder than that of Florida spiny lobster, it is speculated that the PPO activity for these two species could be different. Thus, the objectives of this study were to characterize the PPO from these two lobster species and to compare their kinetic properties to PPOs from other plant and crustacean sources.

Materials and Methods

Fresh Florida spiny lobster (*Panulirus argus*) tails obtained from the Whitney Marine Laboratory at Marineland, FL were transported in ice to the laboratory and stored at -20°C. Frozen Western Australian lobster (*Panulirus cygnus*) tails were purchased from the Beaver Street Foods

(Jacksonville, FL); these lobsters were found to contain less than 5 ppm sulfite background residue when randomly selected samples from each batch were checked using the method of Simpson et al. (1988b). Mushroom (*Agaricus bispora*) tyrosinase with an activity of 2,200 units/mg solid was purchased from Sigma Chemical Co. (St. Louis, MO). Russet potatos and Red Delicious apples were purchased from a local supermarket. White shrimp (*Penaeus setiferus*) and brown shrimp (*Penaeus aztecus*) were obtained from a local seafood store. Grass prawn or Taiwanese black tiger shrimp (*Penaeus monodon*) frozen in dry ice was provided by Dr. J. S. Yang, Food Industry Research and Development Institute, Hsinchu, Taiwan, Republic of China. Lobster cuticle, shrimp cephalothorax (head), and potato and apple peels were frozen in liquid nitrogen and ground into a fine powder using a Waring blender. The ground powder was stored at -20°C until needed.

Extraction and Purification of Lobster PPO

Frozen lobster tails were thawed at room temperature. After the cuticle was separated from the flesh, it was frozen in liquid nitrogen and ground into a fine powder in a Waring blender. The cuticle powder was stored at -20°C and used as required.

PPO was extracted according to the procedure of Simpson et al. (1988a). One part lobster cuticle powder was added to three parts (w/v) 0.05 M sodium phosphate buffer (pH 7.2) containing 1 M NaCl and 0.2% Brij 35 (Fisher Scientific Co., Orlando, FL). The extract was stirred for 3 hr at 4°C and the suspension was centrifuged at 8,000*g* (4°C) for 30 min. The supernatant was then dialyzed at 4°C overnight against 3 changes (4L) of 0.05 M sodium phosphate buffer (pH 6.5).

Each enzyme was purified further using a nondenaturing preparative polyacrylamide gel electrophoresis (PAGE) system. Equipment utilized included a gel tube chamber (Bio-Rad Model 175, Richmond, CA) and a Bio-Rad power supply (Model EPS 500/400). A one-mL aliquot of crude enzyme extract was applied to each of eight gel tubes (1.4 cm I.D. x 12 cm length) containing 5% acrylamide/ 0.13% bisacrylamide gel prepared according to the method of Sigma Bulletin No. MKR-137 (Sigma Chemical Co., 1984), and ran at a constant current of 10 mA/tube in a buffer (pH 8.3) containing 5 mM Tris-HCl and 38 mM glycine. PPO was visualized using a specific enzyme-substrate staining method (Constantinides and Bedford, 1967); 10 mM DL-β-3,4-dihydroxyphenylalanine (DL-DOPA) in 0.05 M sodium phosphate buffer (pH 6.5) was used as a substrate. One tube was used to determine the migration of the enzyme relative to the dye front (R_f). The remaining gels were then sectioned at the determined R_f and the enzyme was eluted from the gel by homogenization in 0.05 M sodium phosphate buffer (pH 6.5) utilizing a Dounce manual tissue grinder (Wheaton, Millville, NJ). The homogenates were filtered through Whatman No. 4 filter papers, pooled, and concentrated using an Amicon stirred cell (Model 8050, Amicon Co., Danvers, MA) fitted with a 10 K filter (Pharmacia LBK Biotechnology Inc., Piscataway, NJ).

Extraction and Purification of Potato PPO

The method of Patil and Zucker (1965) with some modifications was used. After ammonium sulfate precipitation and dialysis, crude PPO preparation was subjected to chromatography with a DEAE-cellulose (0.95 meq/g, Sigma) column (40 cm length x 26 mm i.d., K 26/40 Pharmacia Fine

Chemicals) which had been equilibrated with 1.0 mM potassium phosphate buffer (pH 7.0). Unbound phenolic compounds and proteins were washed off using 250 mL 1 mM phosphate buffer (pH 7.0) at 24 mL/hr for 3 hr. Elution of PPO was performed using a linear gradient (0 - 1.0 M) of NaCl in 1.0 mM potassium phosphate buffer at 24 mL/hr for 18 hr. Four-mL fractions were collected, and protein was estimated by spectrophotometry at 280 nm. Fractions showing PPO activity were pooled and concentrated using an Amicon stirred cell fitted with an Amicon YM 10 filter.

The partially purified enzyme preparation was loaded onto a Sephadex G-100 (Pharmacia) gel filtration column (Pharmacia K 26/40) pre-equilibrated with 1.0 mM potassium phosphate buffer (pH 7.0). The column was eluted at 4°C with 400 mL 1.0 mM potassium phosphate buffer (pH 7.0) at 24 mL/hr for 15 hr. Four-mL fractions were collected and protein estimated as above; fractions showing PPO activity were pooled and concentrated using an Amicon stirred cell. Concentrated samples were dialyzed at 4°C overnight against 3 changes of 2L elution buffer.

Extraction and Purification of Apple PPO

The method of Stelzig et al. (1972) with some modification was followed. Crude apple PPO after partial purification and dialysis against H_2O was loaded onto an HT hydroxylapatite (Bio-Rad) column (K 26/40). The enzyme was desorbed from the gel using 250 mL 0.005 - 0.3 M (linear gradient) sodium phosphate buffer (pH 7.6) containing 5% ammonium sulfate at 24 mL/hr for 18 hr. Four-mL fractions were collected and protein estimated by absorbance at 280 nm; fractions showing PPO activity were pooled and dialyzed overnight (4°C) against 2 changes of 4L H_2O. The

dialysate was further concentrated using an Amicon stirred cell fitted with a YM 10 filter.

Extraction and Purification of White Shrimp and Grass Prawn PPO

The methods of Chen et al. (1991a) and Rolle et al. (1991) with slight modification were followed to purify white shrimp and grass prawn PPO, respectively. Shrimp cephalothorax powder was suspended in 3 volumes (w/v) 0.05 M sodium phosphate buffer (pH 7.2) containing 1 M NaCl (extraction buffer) and 0.2% (v/v) Brij 35, and stirred at 4°C for 3 hr. Following centrifugation at 23,000*g* (4°C) for 30 min, the supernatant was fractionated with ammonium sulfate between 0 - 40% saturation; protein precipitate was collected by centrifugation at 23,500*g* at 4°C for 30 min.

For white shrimp, the precipitate was dissolved in 0.05 M phosphate buffer (pH 7.2) and dialyzed at 4°C overnight against 3 changes of 4L of 0.05 M phosphate buffer (pH 7.2). The dialyzed PPO was loaded onto a DEAE-cellulose (0.95 meq/g) column (K 26/40) pre-equilibrated with 0.05 M phosphate buffer (pH 7.2). Sixty-mL of 0.05 M sodium phosphate buffer (pH 7.2) was used to desorb unbound phenolic compounds and proteins at 0.2 mL/min for 1.5 hr. Elution of PPO was performed using a 300 mL 0.05 M sodium phosphate buffer (pH 7.2) containing a linear gradient (0 - 1.0 M) of NaCl. Three-mL fractions were collected and the protein estimated by absorbance at 280 nm. Fractions possessing PPO activity were pooled and concentrated using an Amicon stirred cell fitted with a YM 10 filter.

PPO was loaded onto a Sephadex G-100 gel column (K 26/40) pre-equilibrated with 0.05 M sodium phosphate buffer (pH 7.2) and then eluted with 300 mL 0.05 M sodium phosphate buffer (pH 7.2) at 0.15 mL/min. Three-mL fractions were collected and protein was estimated by absorbance at 280 nm; fractions showing PPO activity were pooled and concentrated using an Amicon stirred cell fitted with YM 10 filter. Concentrated PPO was then dialyzed at 4°C overnight against 3 changes of 2L H_2O.

For grass prawn, the precipitate was resuspended in extraction buffer containing 40% ammonium sulfate. After homogenization using a Dounce manual tissue grinder, the sample was centrifuged at 23,500*g* (4°C) for 20 min. The precipitate was homogenized in extraction buffer and centrifuged as previously described. The resulting precipitate was homogenized in extraction buffer, then subjected to high performance hydrophobic interaction chromatography at 4°C using a preparative Phenyl Sepharose CL-4B (Sigma) column (K 16/40) attached to a Dionex gradient pump (Dionex Corp., Sunnyvale, CA). The column was pre-equilibrated with extraction buffer.

PPO was eluted with a stepwise gradient of elution buffer [100% extraction buffer (9 mL), 50% extraction buffer in water (24 mL), and 10% extraction buffer in water (24 mL)], 50% ethylene glycol (12 mL), and then distilled water (150 mL) at a flow of 0.2 mL/min. Four-mL fractions were collected and fractions exhibiting PPO activity were pooled and concentrated via ultrafiltration utilizing an Amicon stirred cell fitted with an XM 50 filter (Amicon).

Protein Quantitation and Enzyme Purity Determination

The protein contents of the various PPO preparations were quantitated using the Bio-Rad Protein Assay kit with bovine serum albumin (Sigma) as standard. Enzyme purity was examined using a mini gel system (Mini-Protean II Dual Slab Cell) (Bio-Rad, 1985b). Plant and crustacean PPO's (20 μg protein/well) were loaded and electrophoresis was carried out at constant voltage (200 V) in a buffer (pH 8.3) containing 25 mM Tris-HCl and 0.19 M glycine for 35 min. The purity of enzyme preparations was determined by comparing gels stained with 10 mM DL-DOPA in 0.05 M sodium phosphate buffer (pH 6.5) and then with a Commassie blue R-250 (Eastman Kodak Co., Rochester, NY) solution.

PPO Activity Determination

Lobster PPO activity was determined spectrophotometrically by monitoring at 475 nm the rate of dopachrome formation from DL-DOPA (Savagaon and Sreenivasan, 1978). The assay was run at 25°C for 10 min by mixing 40 μL of enzyme extract with 560 μL of 10 mM DL-DOPA in 0.05 M sodium phosphate buffer (pH 6.5). Two molecules of DOPA produce one molecule of dopaquinone which has a molar absorption coefficient (a_{m475}) of 3,600 $M^{-1}cm^{-1}$ (Fling et al., 1963). PPO activity was defined as μmoles dopachrome formed per min at 25°C.

PPO activities of shrimp, potato, and apple were measured by adding 60 μL enzyme preparations to 840 μL 10 mM DL-DOPA in 0.05 M sodium phosphate buffer (pH 6.5) and monitored at 25°C for 5 min. Maximal initial velocity was determined as $\Delta A_{475\ nm}$/min and one unit of PPO activity was defined as an increase in absorbance of 0.001/min at 25°C. Unless

otherwise specified, experiments were carried out three times in duplicate. For this study, the enzyme activities of potato, apple, spiny lobster, Australian lobster, white shrimp, and grass prawn PPOs were determined to be 10,900, 97,400, 7,000, 3,000, 5,400, and 900 units/mg protein, respectively.

pH Optima and Stability of Lobster PPO

The modified method of Gormori (1955) was followed for preparation of various buffer solutions including 0.1 M sodium citrate-0.1 M HCl, pH 2.0, 3.0, and 4.0; 0.05 M sodium phosphate, pH 5.0, 6.0, 6.5, 7.0, 7.5, and 8.0; 0.1 M glycine-0.1 M NaOH, pH 9.0, and 10.0; and 0.1 M sodium phosphate-0.1 M NaOH, pH 11.0 and 12.0. The assay was performed at 25°C by adding 40 μL enzyme solution to a mixture containing 280 μL of buffer solution and an equal volume of 10 mM DL-DOPA in distilled water. The mean velocity for dopachrome formation was determined at 475 nm using a DU-7 spectrophotometer (Beckman Instruments, Inc., Irvine, CA).

After enzyme mixtures containing 40 μL enzyme preparation and 120 μL of each of the previously described buffer systems were incubated at 25°C for 30 min, a 40 μL aliquot was removed and added to 560 μL of 10 mM DL-DOPA solution in distilled water. Dopachrome formation was monitored spectrophotometrically.

Activation Energy and Thermostability of Lobster PPO

Reaction mixtures containing 40 μL enzyme extract and 560 μL 10 mM DL-DOPA solution were incubated at various temperatures ranging from 20° to 60°C. Activation energy (E_a) was determined according to the Arrhenius

equation by measuring the initial rate of reaction at different temperatures and plotting the logarithmic value of V_{max} versus 1/T (Segel, 1976).

To determine the thermostability, a 40 μL aliquot of lobster PPO was sealed in a quartz cell and incubated in a DU-7 spectrophotometer for 30 min at different temperatures ranging from 20-60°C. Following equilibration to room temperature, the enzyme extract was mixed with 560 μL of 10 mM DL-DOPA and then monitored spectrophotometrically as described above.

Molecular Weight Determination of Lobster PPO

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used for molecular weight determination of the enzyme isoforms. Slab gels (16 cm x 20 cm) at 1.50 mm thickness, consisting of stacking gel (4% acrylamide/ 0.1% bisacrylamide) and separating gel (7.5% acrylamide/ 0.2% bisacrylamide), were prepared according to the Protean™ II Slab Cell Instruction Manual (Bio-Rad Labs., 1985a). Electrophoresis was carried out in a Protean II Slab Cell system equipped with a Bio-Rad Model 3000/300 power supply. A constant current of 13 mA/gel and 18 mA/gel was applied to the stacking and separating gel, respectively in a buffer (pH 8.3) containing 5 mM Tris-HCl and 38 mM glycine. Enzyme samples were diluted with 4 volumes of SDS reducing buffer and then heated at 95°C for 4 min. Fifty-μg aliquots were applied to each sample well. An SDS-6H Molecular Weight Marker Kit (Sigma) containing carbonic anhydrase (29,000), egg albumin (45,000), bovine albumin (66,000), phosphorylase B (97,000), β-galactosidase (116,000), and myosin (205,000) was used. The

molecular weights of the proteins were determined following the methods of Weber and Osborn (1969) and Weber et al. (1972).

Enzyme Kinetics Study

Kinetic parameters (K_m and V_{max}) of the two lobster PPO's were determined using the Lineweaver-Burk equation (Lineweaver and Burk, 1934). DL-DOPA and catechol solutions at concentrations varying from 1.67 to 9.92 mM in 0.05 M phosphate buffer (pH 6.5) were used as substrates. PPO activity on catechol was defined as μmoles of benzoquinone formed per min at 25°C. One molecule of catechol produced one molecule of benzoquinone, which has a molar absorption coefficient (a_{m395}) of 1,350 M^{-1} cm^{-1} (Whitaker, 1972).

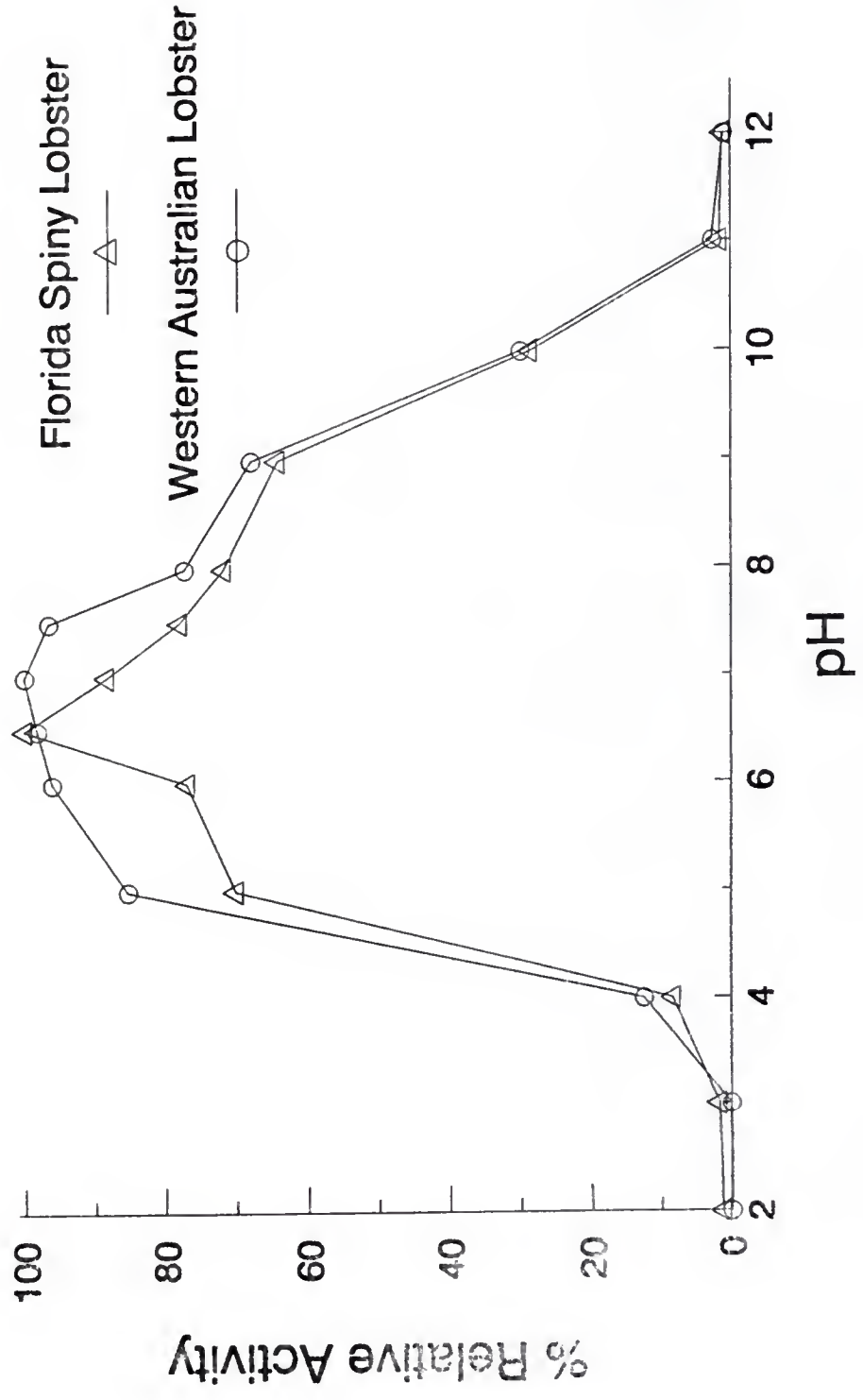
In addition, Michaelis constants (K_m) for mushroom, potato, apple, white shrimp, and grass prawn PPOs were determined. Sixty-μL PPO was added to 10 mM DL-DOPA in 0.05 M sodium phosphate buffer (pH 6.5). The total volume in the cuvette was 1.0 mL and the final concentration of DL-DOPA varied from 1.4 to 8.9 mM. The reaction was monitored at 475 nm and 25°C for 10 min.

Results and Discussion

Effect of pH on Lobster PPO Activity

PPO enzymes isolated from Western Australian lobster and Florida spiny lobster exhibited similar patterns of sensitivity to pH changes. Florida spiny lobster PPO had a pH optimum of 6.5, which was a half unit less than that for Western Australian lobster PPO (Figure 3). These values for the two lobster PPO's were similar to that of gulf (brown)

Figure 3. Effect of pH on the Activity of Polyphenol Oxidase (PPO) Isolated from Florida Spiny Lobster (△) and Western Australian Lobster (○); the Assay Was Performed at 25°C by Adding 40 μL Lobster PPO to a Mixture Containing 280 μL of Buffer Solution and an Equal Volume of 10 mM DL-DOPA in Distilled H_2O.


Florida Spiny Lobster
Western Australian Lobster
% Relative Activity
100
80
60
40
20
0
2
4
6
8
10
12
pH

shrimp (Madero and Finne, 1982). PPO from white shrimp (*Penaeus setiferus*) had a pH optimum at 7.5, while that from pink shrimp (*Penaeus duorarum*) was 8.0 (Simpson et al., 1987, 1988a). Ohshima and Nagayama (1980) showed that catecholase isolated from antarctic krill (*Euphausia superba*) had a pH optimum at 6.5. PPO isolated from grass prawn was shown to have a pH optimum at 6.0 (Rolle et al., 1991). For plant PPO, the pH optimum of 7.0 was observed for mushroom (Dawson and Mager, 1962), apple (Stelzig et al., 1972), and banana (Galeazzi and Sgarbieri, 1978), respectively, while pear PPO was reported to have a pH optimum at 4.0 (Rivas and Whitaker, 1973).

With respect to the pH-related relative activity, the Western Australian lobster PPO had a broader pH range (from 5 to 9) than that observed for the Florida spiny lobster PPO. The behavior of Florida lobster PPO to various pH environments observed in this study was similar to that of IPO1 (inert phenoloxidase) and TAPO1 (trypsin activated phenoloxidase) forms from a study by Ferrer et al. (1989a). For white and pink shrimp PPO, the optimal pH environments ranged from 6-7.5 and 6.5-9.0, respectively (Simpson et al., 1988a).

Data in Table 1 indicated that crustacean (Florida spiny lobster, Western Australian lobster, pink shrimp, white shrimp, brown shrimp, and grass prawn) PPOs had a narrow range of optimum pH between 6 to 8. However, a broad range of pH optimum between 4 and 7 was observed for mushroom and other plant (potato, apple, peach, pear, and banana) PPOs. Aylward and Haisman (1969) proposed that the optimum pH of PPO activity, which usually ranged between pH 4 and 7, varied with enzyme sources and substrates used.

Table 1. Comparison of Kinetic Properties of Polyphenol Oxidase (PPO) from Various Sources

Enzyme source	Substrate specificity	K_m (mM)	pH optima	Temperature optima (°C)	Molecular Masses (kD)	Isoforms
Mushroom	DOPA[1] Catechol[4,5]	0.20[1]	5.5 - 7[2]	--	34.5[3]	4[3]
Potato	Chlorogenic acid[6]	0.06[1]	6.0[7]	22[8]	36 - 540[9]	5[9]
Apple	4-methylcatechol[10]	0.04[1]	4.5/7.0[10]	30[10]	24 - 134[11]	3[11]
Peach	D-catechin[12]	4.20[12]	6.2[13]	--	70 - 90[12]	4[12]
Pear	Pyrocatechol[14]	20.9[14]	4.0[14]	--	--	2[14]
Banana	Dopamine[15]	0.63[15]	7.0[15]	37[15]	12/60[15]	2
S. lobster[1]	DL-DOPA	9.85	6.5	35	82 - 97	3
A. lobster[1]	DL-DOPA	3.57	7.0	30	87 - 92	2
Grass prawn[16]	DL-DOPA	4.45	6.0	45	63 - 80	2
Pink shrimp[17]	DL-DOPA	1.60	8.0	40	40	1
White shrimp[18]	DL-DOPA	2.80	7.5	45	30	1
Brown shrimp[19]	DL-DOPA	--	6.5	--	213	2

[1]Obtained from this study; [2]Dawson and Mager (1962); [3]Bouchilloux et al. (1963); [4]Harrison et al. (1967); [5]Nakamura et al. (1966); [6]Lavollay et al. (1963); [7]Macrae and Dubbleby (1968); [8]Schaller (1972); [9]Matheis and Belitz (1975); [10]Stelzig et al. (1972); [11]Demenyuk et al. (1974); [12]Wong et al. (1971); [13]Luh and Phithakpol (1972); [14]Rivas and Whitaker (1973); [15]Palmer (1963); [16]Rolle et al. (1991); [17,18]Simpson et al. (1987, 1988a); [19]Madero and Finne (1982)

Effect of pH on Lobster PPO Stability

Enzyme stability over a broad pH range (2 - 12) revealed that PPO obtained from both lobster species exhibited optimum stability at pH 7 (Figure 4). When PPO was pre-incubated between pH of 5 and 9, both Florida spiny lobster and Western Australian lobster PPOs still retained at least 60% of their relative activity when compared at pH 6.5. Conformational changes at the active site due to dramatic pH changes may have caused the significant decline in enzyme activity between pH 2 and 5, and between 9 and 12. Similar changes were reported to occur with TAPO2 (trypsin activated phenoloxidase) form of the Florida spiny lobster PPO (Ferrer et al., 1989a). PPO from pink shrimp (Simpson et al., 1988a), white shrimp (Simpson et al., 1987), and grass prawn (Rolle et al., 1991) all exhibited optimal activity within the neutral to alkaline pH range (pH 6-8), and showed maximal stability at pH 8.

Effect of Temperature on Lobster PPO Activity and Stability

Both lobster PPOs showed temperature-related changes in enzyme activity (Figure 5). Results obtained for this study showed that Florida spiny lobster and Western Australian lobster PPO had the temperature optimum at 35° and 30°C, respectively, (Figure 5). These values were lower than those observed for the PPO of pink shrimp (40°C, Simpson et al., 1988a), white shrimp (45°C, Simpson et al., 1987), and grass prawn (45°C, Rolle et al., 1991). Compared to crustacean PPO, the activity of peach PPO was found to increase from 3° to 37°C and then declined up to 45°C (Vamos-Vigyazo, 1981). In apple, the enzyme reached its maximum activity at 30°C when chlorogenic acid was used as substrate (Vamos-Vigyazo, 1981);

Figure 4. Effect of pH on the Stability of Polyphenol Oxidase (PPO) Isolated from Florida Spiny Lobster (△) and Western Australian Lobster (○); Forty-μL PPO Aliquot Removed from the Various Enzyme-buffer Mixture Was Added to 560 μL of 10 mM DL-DOPA in Distilled H_2O and the Assay Was Performed at 25°C.

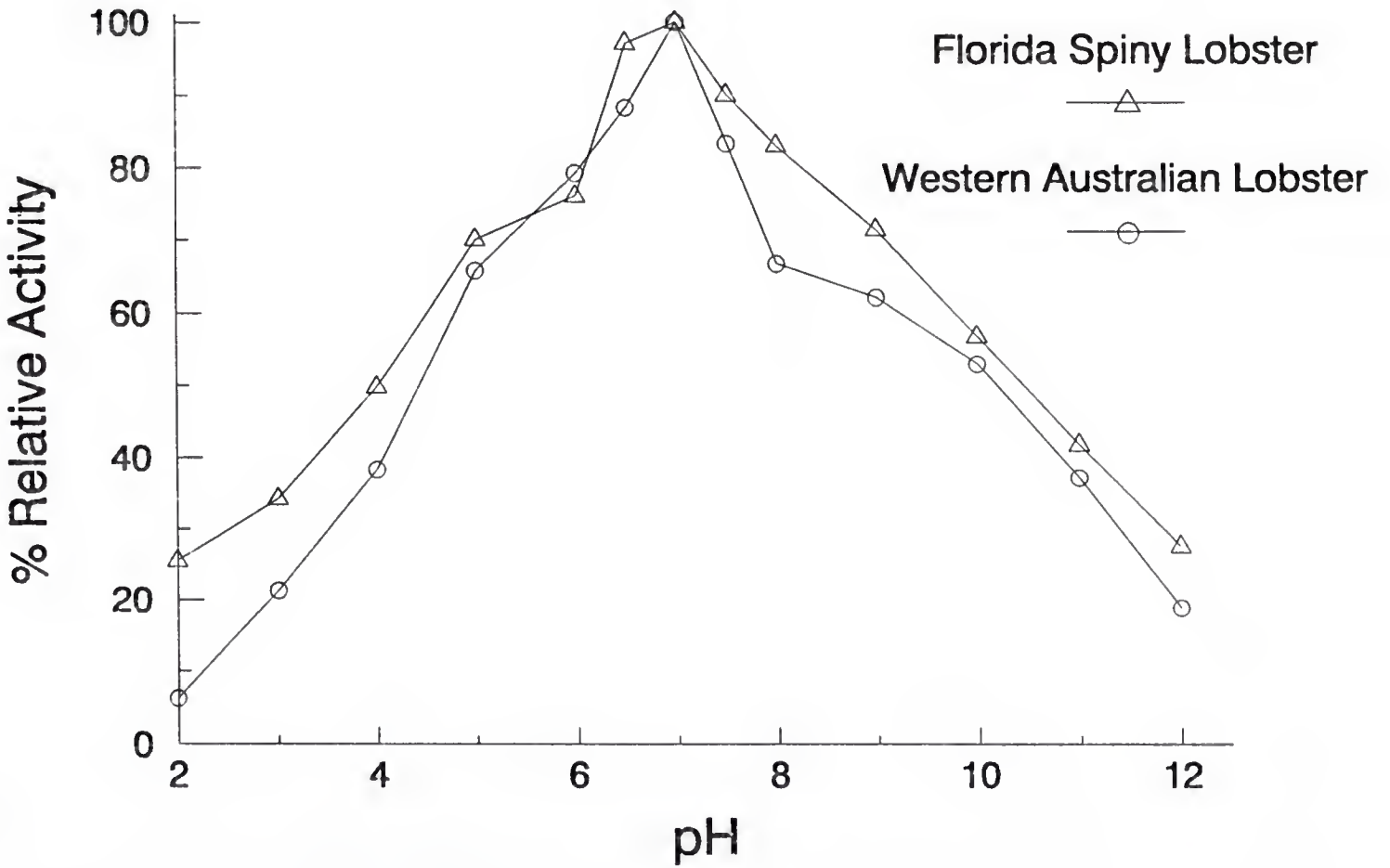
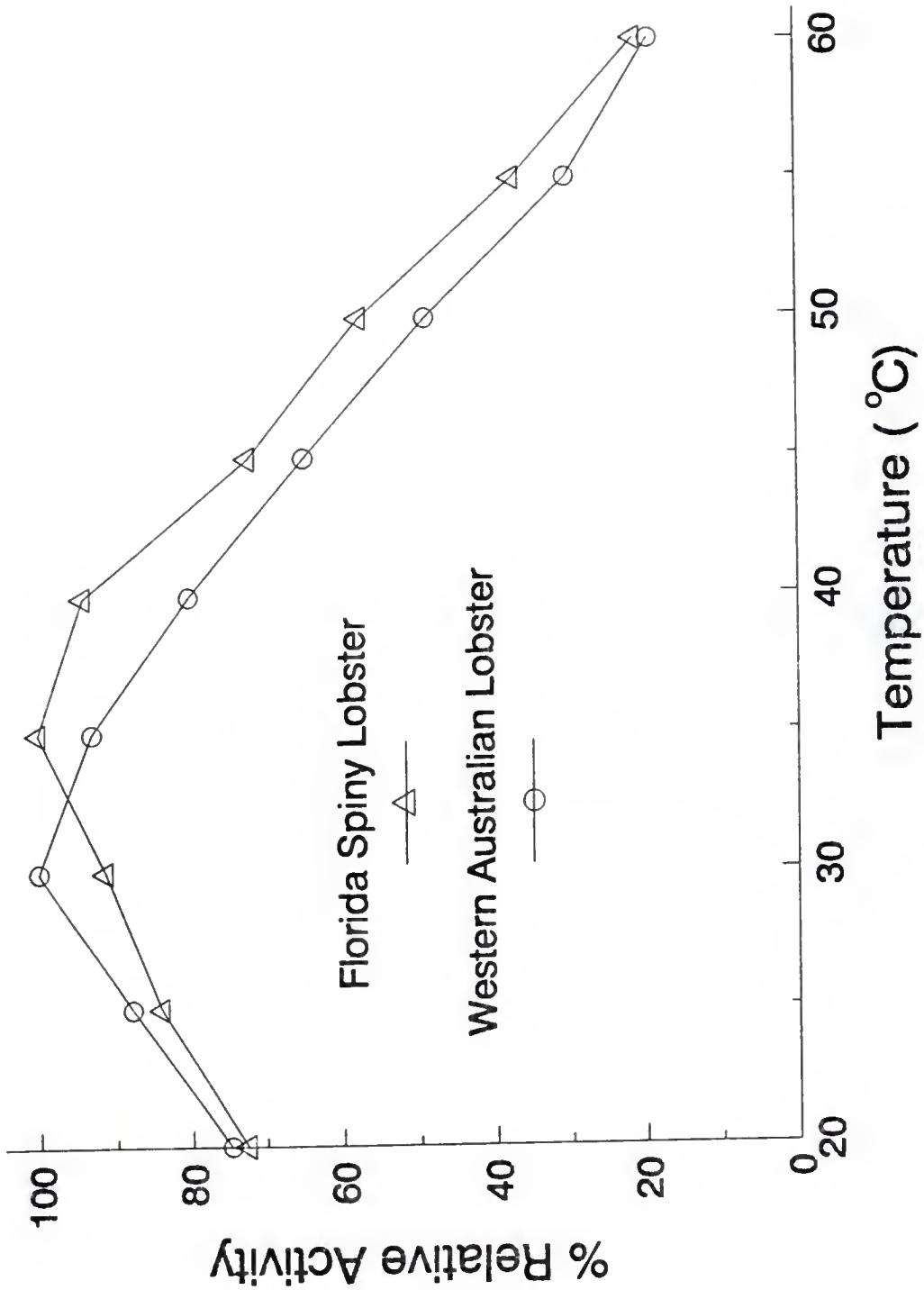

% Relative Activity
100
80
60
40
20
0
2
4
6
8
10
12
pH
Florida Spiny Lobster
Western Australian Lobster

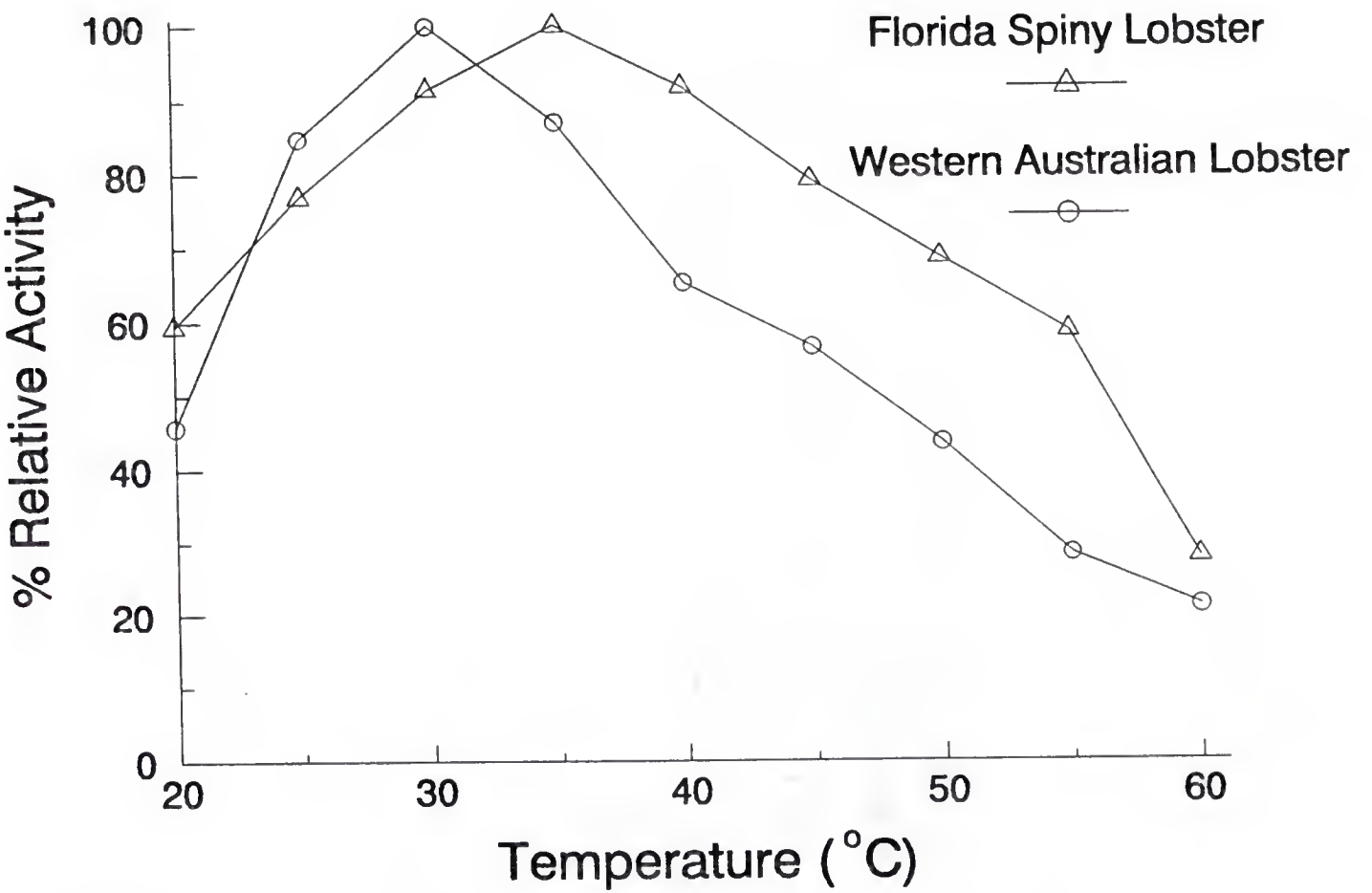
Figure 5. Effect of Temperature on the Activity of Polyphenol Oxidase (PPO) Isolated from Florida Spiny Lobster (△) and Western Australian Lobster (○); the Assay Was Performed by Incubating 560 μL 10 mM DL-DOPA with 40 μL PPO at Various Temperatures.


% Relative Activity
100
80
60
40
20
0
20
30
40
50
60
Temperature (°C)
Florida Spiny Lobster
Western Australian Lobster

; while 37°C was reported for banana PPO using dopamine as substrate (Palmer, 1963). Regarding potato and grape PPO activity, the optimum temperature of 25°C was reported for the former (Lavollay et al., 1963), while 10-15°C for the latter (Cash et al., 1976). The data available indicate that the temperature optimum of the enzyme also depends essentially on the same factors as the pH optimum (Vamos-Vigyazo, 1981). It is noted that most crustacean PPOs had higher temperature optima than those observed for plant PPOs. With the exception of banana PPO, which showed an unusually high temperature optimum at 37°C (Palmer, 1963).

Both lobster PPOs exhibited similar thermostability characteristics, although the Australian lobster PPO showed decreased activity when pre-incubated at temperatures greater than 30°C (Figure 6). However, Florida lobster PPO showed greater stability at a preincubation temperature of 35°C, which was within the range of EAPO (endogenously activated phenoloxidase) and TAPO1 forms but slightly different from the IPO1 form of the Florida spiny lobster PPO (Ferrer et al., 1989a). The thermostability characteristics of both lobster PPO's behave similarly to that of grass prawn (Rolle et al., 1991) and pink shrimp PPO (20-30°C, Simpson et al., 1988a) but varied from that of white shrimp PPO (25-50°C, Simpson et al., 1987). Florida spiny lobsters grew in warm water while Western Australian lobsters are found in cold water. These differences in their habitats may account for the difference in the optimal thermostability between these two enzymes. PPO enzymes from shrimp are usually stable at temperatures ranging between 30° and 50°C (Madero and Finne, 1982; Simpson et al., 1987, 1988a). Most PPO enzymes are heat

Figure 6. Effect of Temperature on the Stability of Polyphenol Oxidase (PPO) Isolated from Florida Spiny Lobster (△) and Western Australian Lobster (○); Following Equilibration to Room Temperature, 40 μL Lobster PPO Incubated at Various Temperatures (20° - 60°C) Was Added to 560 μL of 10 mM DL-DOPA and the Assay Was Performed at 25°C.


Florida Spiny Lobster
Western Australian Lobster
% Relative Activity
100
80
60
40
20
0
20
30
40
50
60
Temperature (°C)

labile; a short exposure of the enzyme to temperatures at 70-90°C is sufficient to cause partial or total irreversible denaturation. Crude PPO isolated from deep sea crab was shown to be inactivated at 70°C (Marshall et al., 1984). Similarly, apple PPO (Walker, 1964) and grape PPO (Vamos-Vigyazo, 1981) were found to be markedly inactivated at temperatures above 70°C. For banana PPO, an exposure to 80°C for 15 min was required to inactivate enzymes completely (Galeazzi and Sgarbieri, 1978). Out of 22 cultivars of different stone fruits, peach PPO was found the least thermostable and the greater heat stable enzyme from plum and cherry was accompanied by higher activity when compared to peach PPO (Dang and Yankov, 1970).

Vamos-Vigyazo (1981) pointed out that the thermotolerance of PPO is dependent on the source of enzyme. In addition, different molecular forms of PPO from the same source may behave differently in thermostabilities.

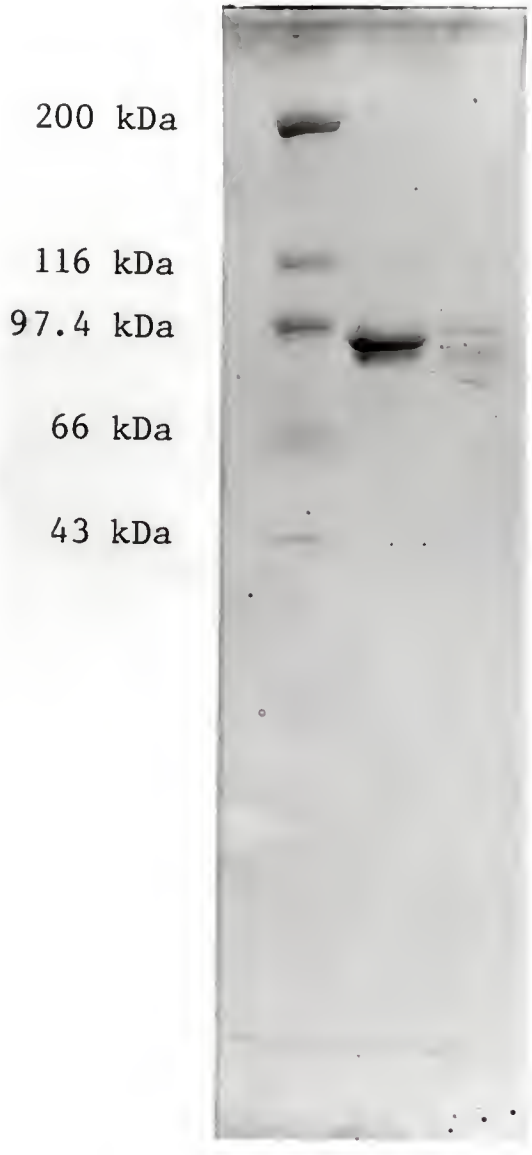
Activation Energy (E_a) of Lobster PPO

The activation energies, E_a, for Florida and Australian lobster PPO were 6.9 and 7.5 Kcal/mole, respectively. These E_a values were similar to TAPO1 (7.8 Kcal/mole) from Florida spiny lobster PPO (Ferrer et al., 1989a), and comparable to shrimp PPO (E_a = 5.2 Kcal/mole) (Bailey et al., 1954), but somewhat different from those of the PPO prepared from white shrimp (E_a = 13.9 Kcal/mole; Simpson et al., 1987), pink shrimp (E_a = 11.5 Kcal/mole; Simpson et al., 1988a), and grass prawn (E_a = 13.3 Kcal/mole; Rolle et al., 1991). The E_a of banana PPO was found to be 4.4 Kcal/mole when catechol was used as substrate (Palmer, 1963).

Molecular Weight and Isoform Determination of Lobster PPO

Figure 7 shows the SDS-PAGE (reduction condition) patterns for both lobster PPO enzymes; the Florida spiny lobster PPO had three isoforms and the Western Australian lobster PPO had two. The molecular masses of the Florida lobster PPO subunits were determined to be 82, 88, and 97 kD, while those of the Australian lobster were 87 and 92 kD. The molecular masses of these subunits were higher than those of white shrimp (30 kD), pink shrimp (40 kD), grass prawn (63 and 80 kD) and antarctic krill (75 and 83 kD) (Simpson et al., 1987, 1988a; Ohshima and Nagayama, 1980; Rolle et al., 1991), but lower than that of brown shrimp (210 kD) (Madero and Finne, 1982). Multiple molecular forms with different molecular masses were observed for PPO from various plant sources (Vamos-Vigyazo, 1981). Mushroom PPO was reported to have 4 isoforms (isozymes), each had molecular masses of 34.5 kD (Bouchilloux et al., 1963). Apple PPO was shown to have 3 isoforms with molecular masses of 24, 67, and 134 kD, respectively (Demenyuk et al., 1974). PPO from banana was reported to possess two isoforms with molecular masses of 12 and 60 kD, respectively (Palmer, 1963). Regarding potato, multiple isoforms of PPO bearing varied molecular masses have been reported by many workers (Anisimov et al.,1978; Constantinides and Bedford, 1967; Patil and Evans, 1963; Thomas et al., 1978). Although Florida spiny lobster and Western Australian lobster were grown in different habitats, a recent study using immunological techniques with rabbit antisera against the Florida spiny lobster PPO revealed that these two lobsters shared cross-reactivity (Rolle et al., 1991).

Figure 7. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE, 7.5% Gel) Profile of Polyphenol Oxidase from Florida Spiny Lobster (FSL) and Western Australian Lobster (WAL); PPO Samples Were Diluted with 4 Volumes of SDS Reducing Buffer Containing β-Mercaptoethanol and Heated at 95°C for 4 Min. Fifty-μg PPO Was Loaded onto Each Sample Well.


200 kDa
116 kDa
97.4 kDa
66 kDa
43 kDa
Protein Standard
WAL PPO
FSL PPO

PPO from most sources, as mentioned previously, have been reported to be present in different molecular forms. The number of these forms depends on the enzyme source and on methods used to prepare them. It has been revealed that part of molecular forms are due to association-dissociation phenomena which is attributed to (1) association of various degrees of polymerization of similar units; (2) various combinations of different subunits; (3) conformational changes of a single protein; or combinations of these three possibilities (Vamos-Vigyazo, 1981). Much attention has been paid to differences in the properties of multiple forms of the enzyme and to the possible physiological significance of such differences. These include differences in affinity and specificity to phenolic substrates and to oxygen (Harel et al., 1964; Kahn, 1976; Taneja and Sachar, 1974), sensitivity to inhibitors (Constantinides and Bedford, 1967; Harel et al., 1964), pH optima (Takeo and Uritani, 1965; Wong et al., 1971), and inactivation by heat (Ben-Shalom et al., 1977; Fling et al., 1963; Sussman, 1961). In addition, differences in isozyme patterns were also reported in connection with subcellular organelles (Harel et al., 1965), the stage of tissue development (Takeo and Baker, 1973; Taneja et al., 1974), as result of attack by pathogens (Hyodo and Uritani, 1964), or of treatment with plant hormones (Taneja and Sachar, 1977a, b).

Enzyme Kinetics of Lobster PPO

Double-reciprocal plots for the oxidation of DL-DOPA and catechol by both lobster PPOs are shown in Figures 8 and 9, respectively. Both lobster PPOs were capable of catalyzing DL-DOPA and catechol. Australian lobster PPO displayed a relatively greater Michaelis constant (K_m = 3.57

Figure 8. Double Reciprocal Plots for the Oxidation of DL-DOPA (●) and Catechol (O) by Florida Spiny Lobster Polyphenol Oxidase (PPO); DL-DOPA or Catechol at Concentrations of 1.67 - 9.92 mM in 0.05 M Sodium Phosphate Buffer (pH 6.5) Was Used as Substrate and the Assay Was Performed at 25°C.

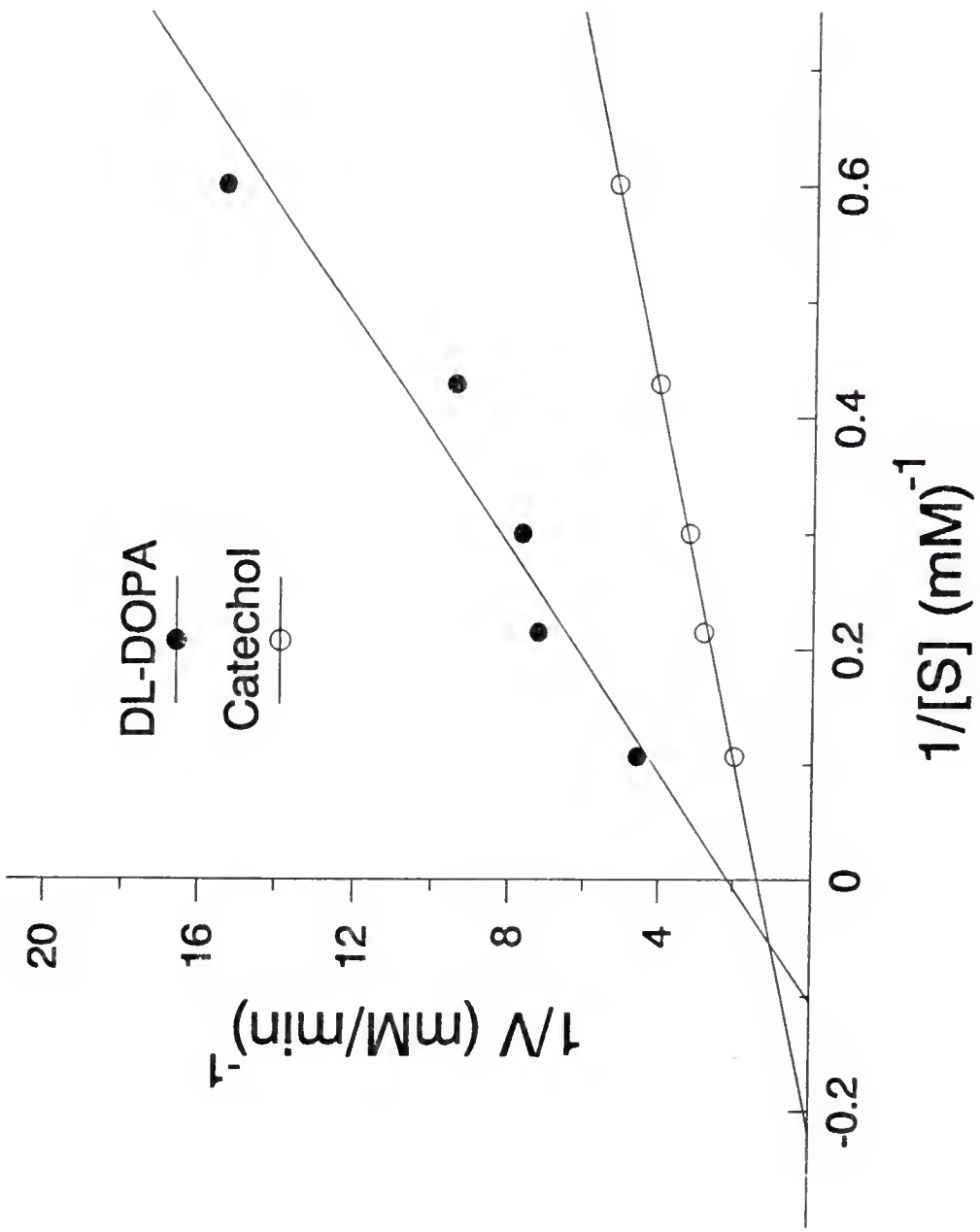
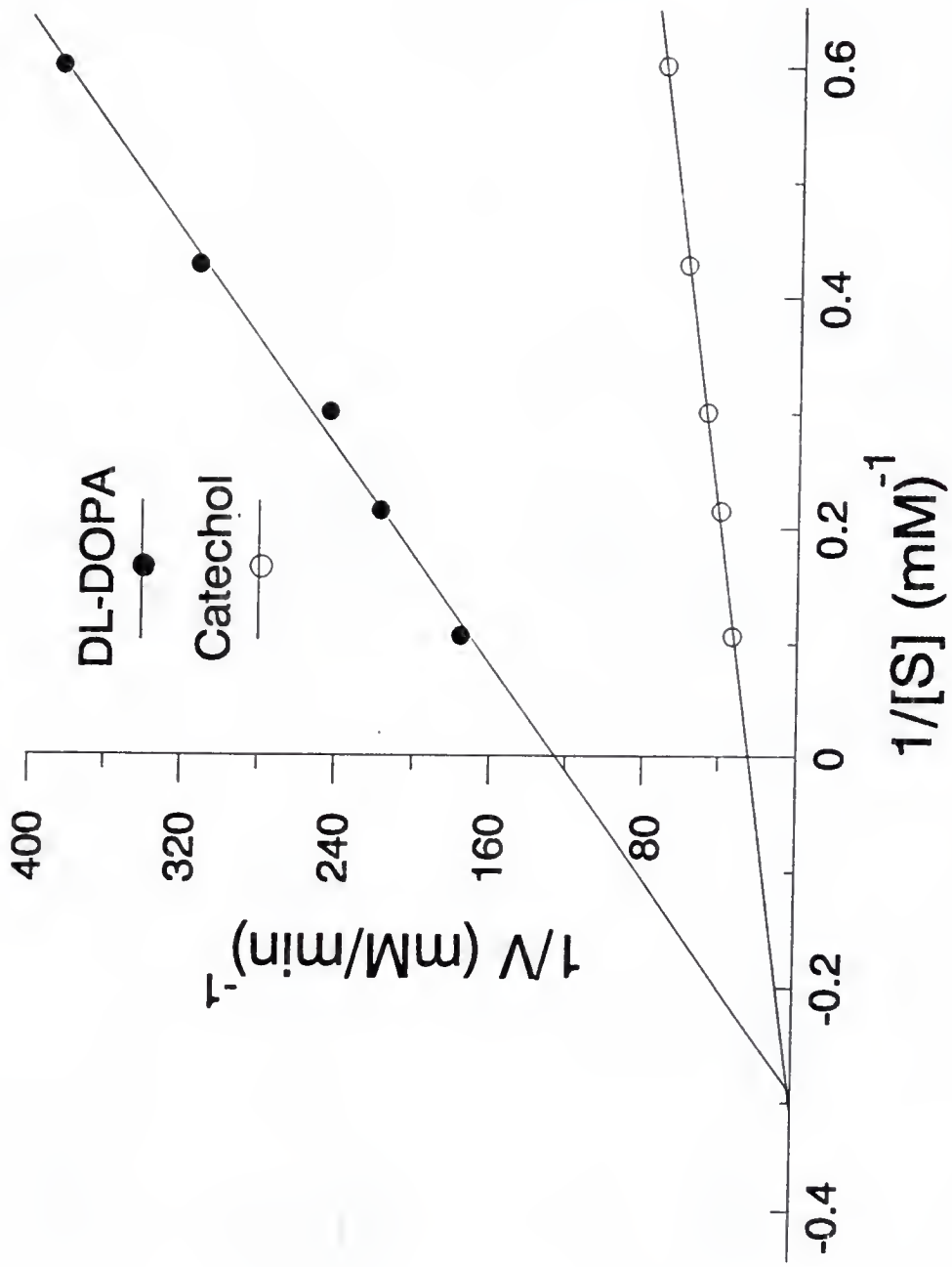

DL-DOPA
Catechol
1/V (mM/min)$^{-1}$
1/[S] (mM)$^{-1}$
20
16
12
8
4
0
-0.2
0.2
0.4
0.6

Figure 9. Double Reciprocal Plots for the Oxidation of DL-DOPA (●) and Catechol (O) by Western Australian Lobster Polyphenol Oxidase (PPO); DL-DOPA or Catechol at Concentrations of 1.67 - 9.92 mM in 0.05 M Sodium Phosphate Buffer (pH 6.5) Was Used as Substrate and the Assay Was Performed at 25°C.


DL-DOPA
Catechol
1/V (mM/min)-1
400
320
240
160
80
0
-0.2
-0.4
0.2
0.4
0.6
1/[S] (mM)-1

mM) and a lower maximum velocity (V_{max} = 0.008 mmol/liter/min) with DL-DOPA than with catechol (K_m = 3.09 mM and V_{max} = 0.041 mmol/liter/min) (Table 2). Similar results were also noted for the Florida lobster PPO (Table 2). These two PPOs had a higher affinity for catechol than for DL-DOPA. Using DL-DOPA as a substrate, Simpson et al. (1987, 1988a) showed that pink shrimp PPO had a lower Michaelis constant (1.6 mM) than white shrimp (2.8 mM). Rolle et al. (1991) characterized grass prawn PPO and they determined the K_m of enzyme was 4.45 mM when DL-DOPA was used as substrate. Summers (1967) showed that blood PPO from fiddler crab had a K_m value of 0.50 mM.

Australian lobster PPO exhibited a higher affinity for DL-DOPA and catechol than Florida lobster PPO. However, the latter showed a greater rate for oxidizing DL-DOPA and catechol than the former. It has been reported by Lavollay et al. (1963) that no relationship could be found between K_m and V_{max} values obtained for a substrate with a given PPO preparation. Instead, the term of V_{max}/K_m has been recommended by some authors (Lavollay et al., 1963; Pollock, 1965) to express the efficiency of a given substrate for a given enzyme. Table 2 indicates that Florida lobster PPO not only showed a higher specific activity but also had a higher turnover number than the Australian lobster PPO. The Florida lobster PPO also showed a higher physiological efficiency for both substrates than the Australian lobster PPO (Table 2), which could account for why Florida spiny lobsters were more susceptible to melanosis than Western Australian lobsters. The kinetic and molecular properties of PPO from other sources are also summarized in Table 1. With DL-DOPA as substrate, different Michaelis constants (K_m) were observed for mushroom,

Table 2. Comparison of Polyphenol Oxidase (PPO) Activity between Florida Spiny Lobster and Western Australian Lobster

PPO	Specific activity[a] (ΔA/min/mg protein)	Turnover[a] No.	V_{max} (mmole/liter/min)		K_m (mM)		V_{max}/K_m (min^{-1})[b]	
			DOPA[a]	Catechol	DOPA[a]	Catechol	DOPA[a]	Catechol
Florida Spiny Lobster	0.36	5.3×10^7	0.48	0.72	9.85	4.58	0.05	0.16
Western Australian Lobster	0.03	4.5×10^6	0.01	0.04	3.57	3.09	0.01	0.01

[a]Spiny lobster and Australian lobster PPO with activity of 7,000 and 3,000 units/mg protein, respectively, were used for this study; DL-DOPA was used as substrate and the unit of turnover number was expressed as μmoles DL-DOPA/min/mole of lobster PPO.

[b]Physiological efficiency

potato, apple, grass prawn, pink shrimp, and white shrimp PPOs. Varied K_m values were also reported for peach (K_m = 4.2 mM, Wong et al., 1971), pear (K_m = 20.9 mM, Rivas and Whitaker, 1973), and banana (K_m = 0.63 mM, Palmer, 1963) PPOs when different phenolic compounds were used as substrates (Table 2). Data obtained from this study showed that mushroom, potato, and apple PPOs had comparatively lower K_m than those of crustacean PPOs, indicating that the former PPOs had a higher affinity for DL-DOPA than the latter ones. It has been reported that the affinity of PPO towards a given substrate may vary widely, even if isozymes of the same origin are used. The differences might be due, at least partly, to steric factors connected to differences in protein structure (Vamos-Vigyazo, 1981).

Substrate specificity varies considerably for PPO from various sources (Aurand and Wood, 1977; deMan, 1985). Simpson et al. (1988a) identified higher levels of free tyrosine and phenylalanine as natural substrates of melanosis in the carapace of pink shrimp. Further studies by Ali (1991) showed PPO isolated from Florida spiny lobster was capable of hydroxylating free tyrosine to DOPA. Tyramine, produced by bacterial activity on free L-tyrosine (Veciana-Nogues et al., 1989; Santos-Buelga et al., 1986) was also identified as the substrate of crab PPO (Summers, 1967). For mushroom (*Agaricus bisporum*) PPO, DOPA and catechol were, respectively, reported by Bouchilloux et al. (1962) and Nakamura et al. (1966) as specific substrates. For potato and apple PPOs, chlorogenic acid (Patil and Zucker, 1965) was considered a specific substrate for the former, while 4-methylcatechol for the latter (Stelzig, 1972). Compared to pear PPO with pyrocatechol (Rivas and Whitaker, 1973) and banana PPO with dopamine (Palmer, 1963), D-catechin was demonstrated as the specific

substrate of peach (Luh and Phithakpol, 1972). Results from this study show that DOPA was the unanimous substrate of crustacean PPOs. For mushroom and other plant PPOs, the specific substrate varied with the sources of enzyme. Mason (1955) reported that tyrosine and DOPA were specific substrates of animal tissue PPO, while Mayer and Harel (1979) listed a wide variety of mono- and *o*-diphenols as the substrates of fungal and plant PPOs. Besides the previously discussed characteristics (substrate specificity, K_m, pH and temperature effects, enzyme isoforms, and molecular weights), other biochemical properties concerning the catalytical function (mechanism), response to the activator/inhibitor, and isoelectric profile (pI) of PPO also varied with the enzyme sources.

Conclusion

PPO isolated from Florida spiny lobster and Western Australian lobster showed very similar patterns in response to effects of pH and temperature on enzyme activity and in SDS-PAGE profile. Using DL-DOPA and catechol as substrates, the Western Australian lobster PPO was shown to have higher affinity but lower physiological efficiency than the Florida spiny lobster PPO. These two PPOs showed distinctly different properties for catalyzing the oxidation of phenolic substrates; this may explain differences in susceptibility of spiny lobster to melanosis compared to Western Australian lobster. Results indicate that PPO from various plant and crustacean sources vary in substrate specificity, kinetic properties, molecular weights, isoforms, activity and stability to pH and temperature effects, activation energy, and isoelectric profiles.

STRUCTURAL COMPARISON OF CRUSTACEAN, PLANT, AND MUSHROOM POLYPHENOL OXIDASES

Introduction

Polyphenol oxidase (PPO) (E.C. 1.14.18.1.), also known as tyrosinase, polyphenolase, phenolase, catechol oxidase, cresolase, and catecholase, is widely distributed in nature (Schwimmer, 1981). The unfavorable enzymatic browning caused by PPO on the surface of many plants and seafood products has been of a great concern to food processors and scientists. Although the formation of melanin (blackening spot) does not affect the nutrient content of food products, it does however connote spoilage by consumers (Eskin et al., 1971). Economic loss resulting from this action has caused great concern among food processors. Enzymatic browning of fruits, vegetables, and crustaceans due to PPO activity has been extensively studied (Chen et al., 1991a; Ferrer et al., 1989a; Flurkey and Jen, 1978; Macrae and Duggleby, 1968; Sciancalepore and Longone, 1984; Simpson et al., 1989a; Walker, 1964).

Differences in the secondary structure between endogenously activated (EAPO) and trypsin activated (TAPO) forms of Florida spiny lobster PPO was recently demonstrated by Rolle et al. (1991) using circular dichroism (CD) spectropolarimetry. A study conducted by Chen et al. (1991b) showed that PPOs from plant and crustacean sources not only varied with respect to catalytic activity in the oxidation of DL-β-3,4-dihydroxyphenylalanine (DL-DOPA) but also had different sensitivities to

the inhibitor, kojic acid (5-hydroxy-2-hydroxymethyl-γ-pyrone). Since the conformational structures of these fungal, plant and crustacean PPOs has not been well documented, this study was undertaken to elucidate whether conformational differences exists among these PPOs using immunological techniques and circular dichroism spectropolarimetry.

Materials and Methods

Fresh Florida spiny lobster (*Panulirus argus*) tails obtained from the Whitney Marine Laboratory at Marineland, FL, were transported in ice to the laboratory and stored at -20°C. Mushroom (*Agaricus bispora*) tyrosinase with an activity of 2,200 units/mg solid was purchased from Sigma Chemical Co. Russet potato was purchased from a local supermarket. White shrimp (*Penaeus setiferus*) and brown shrimp (*Penaeus aztecus*) were obtained from a local seafood store. Lobster cuticle, shrimp cephalothorax (head), and potato peel were each frozen in liquid nitrogen and ground into a fine powder using a Waring blender. The individual ground powder was stored at -20°C until needed.

Extraction of Mushroom, Potato, Lobster, and Shrimp PPO

PPO was extracted according to the procedure of Simpson et al. (1988a). One part ground powder was added to three parts (w/v) 0.05 M sodium phosphate buffer (pH 7.2) containing 1 M NaCl and 0.2% Brij 35. The extract was stirred for 5 min at 4°C and the suspension was centrifuged at 8,000*g* (4°C) for 30 min. The supernatant was then dialyzed at 4°C overnight against 3 changes of 4L 0.05 M sodium phosphate buffer (pH 6.5).

Purification of Mushroom, Potato, Lobster, and Shrimp PPO

Crude PPO preparation was purified further using a nondenaturing preparative polyacrylamide gel electrophoresis (PAGE) system. Equipment utilized included a gel tube chamber (Model 175, Bio-Rad Labs.) and a power supply (Model EPS 500/400, Pharmacia LKB Biotechnology Inc.). A one-mL aliquot of crude enzyme extract (potato, lobster, or shrimp) was applied to each of eight gel tubes (1.4 cm I.D. x 12 cm length) containing 5% acrylamide/ 0.13% bisacrylamide gel and subjected to a constant current of 10 mA/tube in a buffer (pH 8.3) containing 5 mM Tris-HCl and 38 mM glycine (Sigma Chemical CO., 1984). PPO was visualized using a specific enzyme-substrate staining method (Constantinides and Bedford, 1967); 10 mM DL-DOPA in 0.05 M sodium phosphate buffer (pH 6.5) was used as substrate. After the migration of the enzyme relative to the dye front (R_f) was determined using one of the eight gels, the remaining gels were sectioned at the determined R_f. PPO was eluted from the gel by homogenization in 0.05 M sodium phosphate buffer (pH 6.5) utilizing a Dounce manual tissue grinder. The homogenates were filtered through Whatman No. 4 filter paper, pooled, and concentrated using an Amicon stirred cell (Model 8050). Mushroom PPO (0.25 mg/mL) in 0.05 M sodium phosphate buffer (pH 6.5) was further purified according to the procedures previously described.

Protein Quantitation and Enzyme Purity Determination

Protein content of the various PPO preparations was quantitated using the Bio-Rad Protein Assay kit with bovine serum albumin as standard. Enzyme purity was examined using a mini gel system (Mini-Protean II Dual Slab Cell) (Bio-Rad, 1985b). Mushroom, potato, and crustacean PPOs (20 μg

protein/well) were loaded and electrophoresis was carried out at constant voltage (200 V) in a buffer (pH 8.3) containing 25 mM Tris-HCl and 0.19 M glycine for 35 min. Purity of the preparations was determined by comparing gels stained with 10 mM DL-DOPA in 0.05 M sodium phosphate buffer (pH 6.5) and then with a Coomassie blue R-250 solution.

Enzyme Activity Assay

PPO activities were measured by adding 60 μL PPO to 840 μL 10 mM DL-DOPA in 0.05 M sodium phosphate buffer (pH 6.5) and monitoring at 25°C for 5 min in a Beckman DU7 spectrophotometer at 475 nm. Maximal initial velocity was determined as $\Delta A_{475\ nm}$/min and one unit of PPO activity was defined as an increase in absorbance of 0.001/min at 25°C. Unless otherwise specified, experiments were carried out twice in triplicates. For this study, the enzyme activities of mushroom, potato, lobster, white shrimp, and brown shrimp PPO were determined to be 96,000, 120,000, 4,500, 1,000, and 1,200 units/mg protein, respectively.

Anti-lobster PPO Antibody Production and Purification

One-mL purified lobster PPO containing 100 μg protein was used as an antigen to inject into a hen biweekly. Eggs laid by the immunized hen were collected and anti-lobster PPO antibody was isolated and purified from the egg yolk using the method of Polson et al. (1985). One part egg yolk was added to 4 parts (v/v) 0.1 M sodium phosphate buffer (pH 7.6). The mixture was made up 3.5% (w/v) with polyethyleneglycol (PEG) and stirred for 5 min. Following centrifugation at 5,000*g* (10°C) for 20 min, the supernatant collected was made up with 8.5% (w/v) PEG. The suspension

was allowed to stand for 10 min followed by centrifugation at 5,000*g* (10°C) for 25 min. The pellet was dissolved in 2.5 volumes (v/v) phosphate buffer (pH 7.6) and the suspension was made up with PEG to 12% (v/v). Again, the suspension was allowed to stand for 10 min and then centrifuged at 5,000*g* (10°C) for 25 min. The pellet was resuspended in 1/4 volume phosphate buffer and cooled to 0°C before adding an equivalent volume of 50% ethanol (-20°C). Following centrifugation at 10,000*g* (4°C) for 25 min, the precipitate was dissolved in 1/4 volume phosphate buffer and the suspension was dialyzed overnight (4°C) against 4L 0.1 M sodium phosphate buffer (pH 7.6). After dialysis, the antibody preparation was made up with NaN_3 to 0.1% and stored in the refrigerator until needed.

Molecular Weight Determination of Anti-lobster PPO Antibody

Protein content of the antibody preparation was also quantitated using the Bio-Rad Protein Assay kit. The molecular weight of the antibody preparation was determined using SDS-PAGE (reduction condition) according to the method of Laemmli (1970). Mini slab gels (7 cm x 8 cm) at 1.0 mm thickness, consisting of stacking gel (4% acrylamide/ 0.1% bisacrylamide) and separating gel (7.5% acrylamide/ 0.2% bisacrylamide) were prepared according to the Mini-Protein II Dual Slab Cell Instruction Manual (Bio-Rad, 1985b). Antibody preparations were diluted with 4 volumes of SDS sample buffer containing β-mercaptoethanol, and heated for 4 min at 95°C. After 30 μg aliquots were applied to each sample well and electrophoresis was carried out for 35 min at a constant voltages of 200 V. An SDS-6H high Molecular Weight Protein Standards kit (Sigma) containing carbonic anhydrase (29,000), egg albumin (45,000), bovine albumin (66,000),

phosphorylase (97,400), β-galactosidase (116,000), and myosin (205,000) was used. Molecular weights of the antibody proteins were determined following the methods of Weber and Osborn (1969) and Weber et al. (1972).

Antibody Titer Determination by Enzyme-linked Immunosorbent Assay (ELISA)

One hundred-μL lobster PPO containing 2.5 - 100 ng protein in 0.1 M $NaHCO_3$, pH 8.6, (coating buffer) was applied to the sample well of a microplate (Immulon 2, Dynatech). Following overnight incubation at 4°C, the well was aspirated and washed 4 times with PBS-Tween [0.01 M sodium phosphate, pH 7.4, containing 0.15 M NaCl and 0.2% (v/v) Tween 20] using the Nunc-Immuno Wash (A/S NUNC, Denmark). After 100 μL primary antibody (anti-lobster PPO antibody) at amounts of 0.01 - 10 μg in PBS-Tween was added to the well, incubation was allowed to proceed at ambient temperature for 1 hr. The aspirations and washings were repeated as previously described before 0.1 mL secondary antibody (antichicken IgG-alkaline phosphatase conjugate, Sigma) was added. Following another one-hour incubation, the microplate was aspirated and washed again with PBS-Tween. Following the addition of 0.1 mL *p*-nitrophenyl phosphate disodium (1.0 mg/mL) in assay buffer (0.05 M Na_2CO_3 and 0.05 M $NaHCO_3$ containing 0.0005 M $MgCl_2$) to the well, the plate was incubated at ambient temperature till a yellow color developed. The absorbance of the plate at 405 nm was monitored every hour using an ELISA reader (Model 2550, Bio-Rad). In this study, coating buffer without antigen was used as the negative control.

Analysis of Antigenic Properties of PPO

The competitive ELISA adopted from Seymour et al. (1991) was employed to study whether PPO from mushroom, potato, white shrimp, and brown shrimp possessed similar antigenic determinants as the Florida spiny lobster. After the microplate was coated with lobster PPO at 10 ng/well for one hour at room temperature, 100 μL aliquot of antibody-competitive PPO mixture was added. The antibody-PPO mixture was prepared by mixing the competitive PPO (either mushroom, potato, or lobster, white shrimp, and brown shrimp) (0.2 - 2.0 μg/mL) with an equal volume of primary antibody solution (10 or 20 μg/mL of IgY) for 1 hr at ambient temperature. The assay procedures were conducted as previously described.

Immunoblotting

Purified mushroom, potato, lobster, white shrimp, and brown shrimp PPO and a crude lobster PPO preparation were subjected to SDS-PAGE under reduction condition (Laemmli, 1970) and then electro-transferred to nitrocellulose membrane (Bio-Rad). Slab gels (16 cm x 20 cm) at 1.0 mm thickness, consisting of stacking gel (4% acrylamide/ 0.1% bisacrylamide) and separating gel (7.5% acrylamide/ 0.2% bisacrylamide), were prepared according to the Protean™ II Slab Cell Instruction Manual (Bio-Rad, 1985a). A constant current of 13 mA/gel was applied during stacking and 18 mA/gel during separation. Fifty-μg aliquots of test samples were applied to each well and run with the protein standards (SDS-6H Molecular Weight Marker Kit).

Following electrophoresis, the gel was equilibrated in 500 mL of Towbin transfer buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, and 20%

methanol; pH 8.3) for 15 min. Electro-transfer was performed according to the Trans-Blot Electrophoretic Transfer Cell Instruction Manual (Bio-Rad, 1989) at a constant voltage of 50 V for 1.5 hr using Towbin buffer as the electrolytic buffer. Complete transfer of proteins was verified by staining the gel and the nitrocellulose membrane with a Coomassie blue solution.

The nitrocellulose membrane following electro-transferring was rinsed with phosphate-buffered saline (PBS) 3 times, and then incubated with Blotto/Tween blocking solution (5% w/v nonfat dry milk, 0.2% v/v Tween 20, and 0.02% w/v NaN_3 in PBS; Harlow and Lane, 1988) at ambient temperature with agitation for 2 hr. After washing twice for 5 min each in PBS, the membrane was incubated overnight in the primary antibody (IgY) solution (10 μg/mL). Following washing with 4 changes of PBS for 5 min each, the membrane was treated for 1 hr with the secondary antibody (antichicken IgG-alkaline phosphatase conjugate, Sigma) at a dilution of 1/2000. The membrane was then washed again with PBS as previously described, and incubated with 100 mL alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, and 100 mM Tris; pH 9.5) containing 0.016% bromochloroindolyl phosphate (BCIP) and 0.032% nitro blue tetrazolium (NBT) (Harlow and Lane, 1988) for 45 min. The reaction was stopped by rinsing the membrane with PBS containing 20 mM EDTA.

Spectropolarimetric Analysis of PPO

The circular dichromic spectra of PPO was scanned at the far UV (250 - 200 nm) range using a Jasco J-20 automatic recording spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan). A 1.0-cm Suprasil (Helma Cells)

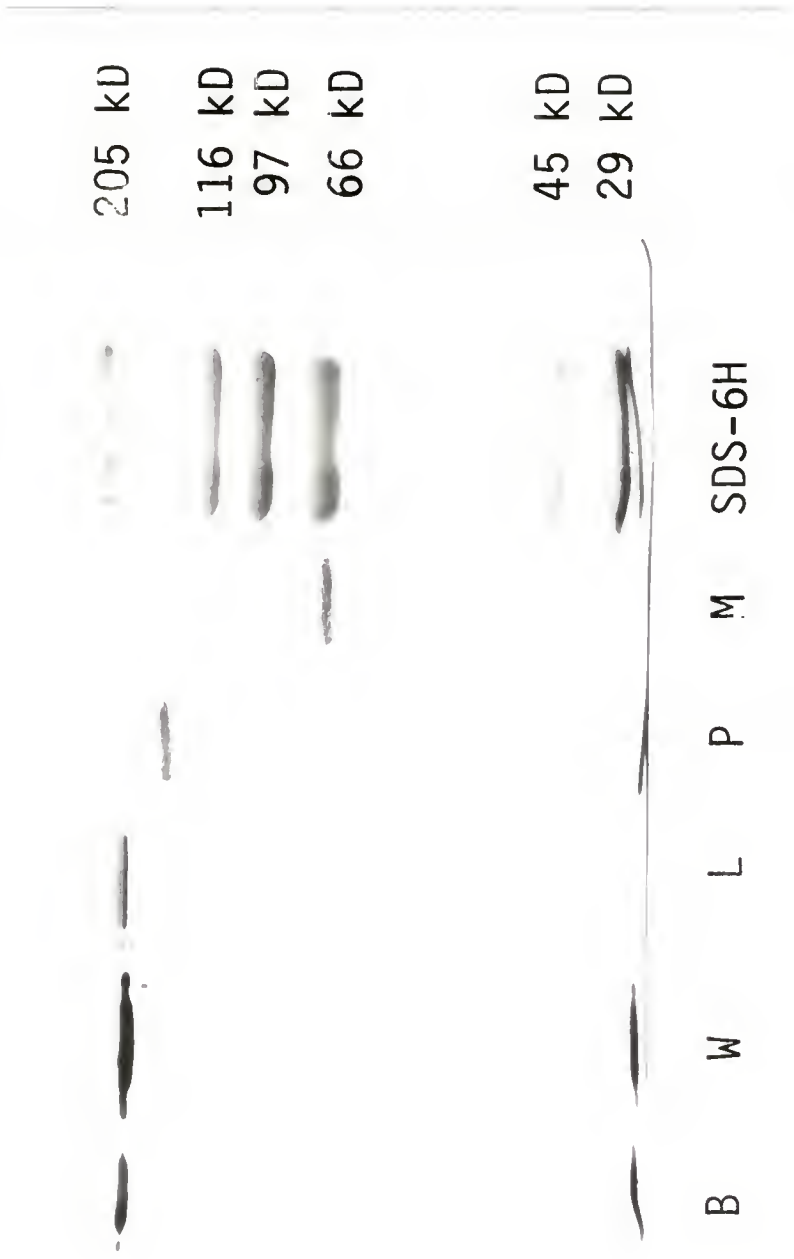
cuvette with a 1.0-cm light path was used. Four-mL of PPO (10 - 20 μg/mL) in 0.5 mM sodium phosphate buffer (pH 6.5) was analyzed at ambient temperature. Calculations of the secondary structures were carried out by computer analysis of the spectra using the SSE program (Japan Spectroscopic Co., 1985) with myoglobin, cytochrome c, ribonuclease A, lysozyme, and papain as references.

Results and Discussion

SDS-PAGE Profile of PPO

Various enzyme subunits with different molecular masses were observed for crude mushroom (7 isozymes), potato (5 isozymes), lobster (3 isozymes), white shrimp (2 isozymes), and brown shrimp (2 isozymes) PPO preparations after the nondenaturing preparative polyacrylamide gel was stained with DL-DOPA. After subjecting to SDS-PAGE (without treatment of β-mercaptoethanol), mushroom and potato PPO chosen for this study were shown to have a subunit of lower molecular mass than crustacean (lobster, white shrimp, and brown shrimp) PPO, estimated as 66 and 148 kD, respectively (Figure 10). These are in agreement with the previously reported data of Anisimov et al. (1978) and Bouchilloux et al. (1963). As to lobster, white shrimp, and brown shrimp PPO, the molecular masses were determined as 200, 190, and 190 kD, respectively. Brown shrimp PPO of 190 kD was close to that of the Gulf brown shrimp PPO (210 kD) (Madero and Finne, 1982). However, the molecular mass of white shrimp PPO determined in this study varied with that reported previously by Simpson et al. (1988). The use of different preparation and analytical methods in these studies could have contributed to such discrepancy.

Figure 10. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE, 7.5% Gel) Profile of Polyphenol Oxidase from Mushroom (M), Potato (P), Florida Spiny Lobster (L), White Shrimp (W), and Brown Shrimp (B) and High Molecular Weight Protein Standards (SDS-6H); PPO Preparations Diluted with 4 Volumes of SDS Sample Buffer without β-Mercaptoethanol Was Heated at 95°C for 4 Min. Twenty-μg PPO Aliquots Was Loaded onto Sample Well.


205 kD
116 kD
97 kD
66 kD
45 kD
29 kD
B
W
L
P
M
SDS-6H

Antibody Production and Molecular Weight Determination

The production of lobster PPO specific antibody did not occur until after the second boosting; peak activity occurred on day 18 (Figure 11). Antibody activity decreased gradually with time following the third and fourth boostings. The serum eventually lost antibody activity after day 35. The animals apparently had become tolerant to the antigen after the third boosting.

Antibody produced on day 17 and 18 having the highest activities was pooled for molecular weight determination. Three distinct bands were observed on the SDS-PAGE gel (Figure 12). The molecular weight for the lower band was estimated as 59,000 which was close to the value reported for the heavy chain of IgY (Jensenius et al., 1981).

Antibody Titer Determination

Dose-related interactions of various PPO (mushroom, potato, lobster, white shrimp, and brown shrimp) at 2.5 - 10 ng with lobster PPO-specific antibody at 2.5 μg are shown in Figure 13. All test samples showed affinity for the antibody. Therefore, these antigens were partially cross-reactive and shared similar antigenic determinants. The appropriate dose range of the interaction of the various PPOs with the antibody was determined to be 2.5 - 10 ng/well. When PPO was added at more than 10 ng/well, the antigen-antibody reaction was saturated and the sensitivity of the ELISA became dull.

Figure 11. Profile of Anti-lobster PPO Antibody Produced from the Immunized Hen during the Immunization Period; Purified Lobster PPO (100 μg Protein) Used as Antigen Was Biweekly Injected into the Animal.

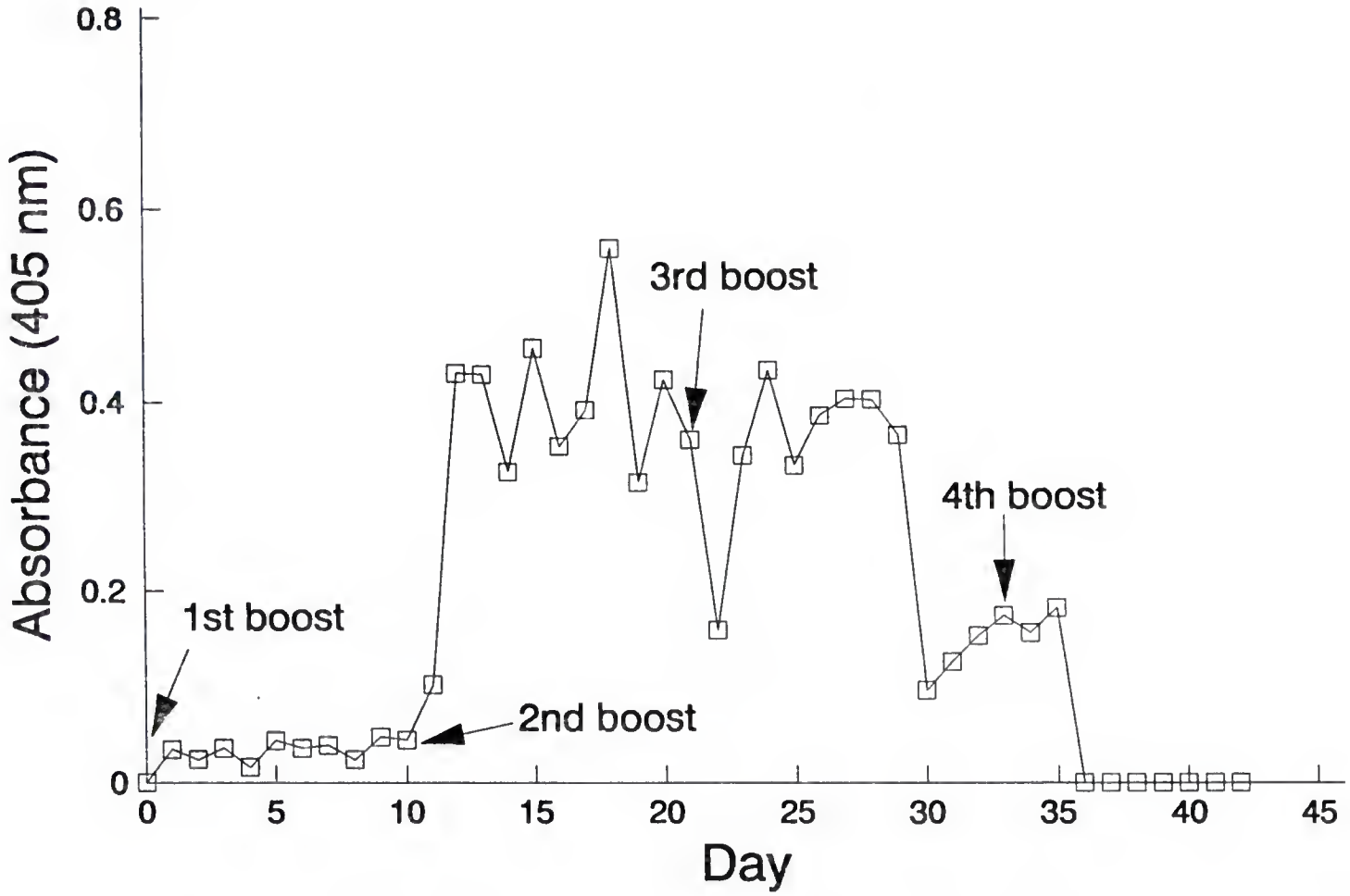

Absorbance (405 nm)
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
30
35
40
45
Day
1st boost
2nd boost
3rd boost
4th boost

Figure 12. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE, 7.5% Gel) of Anti-lobster PPO Antibody and Protein Standard (SDS-6H); Anti-lobster PPO Antibody Was Diluted with 4 Volumes of SDS Sample Buffer Containing β-Mercaptoethanol and Heated at 95°C for 4 Min. Thirty-μg Antibody Preparation Was Loaded onto Each Sample Well.

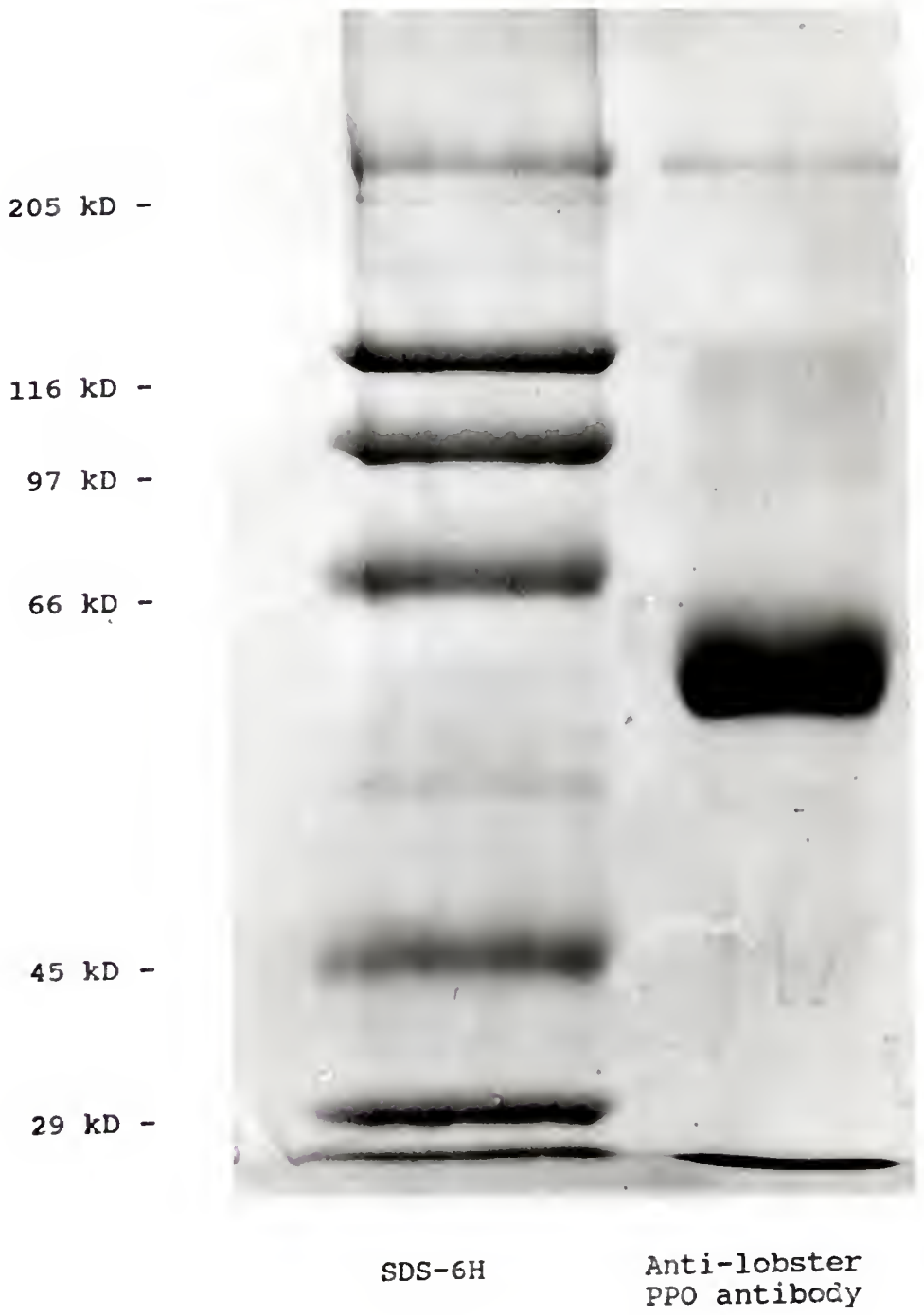
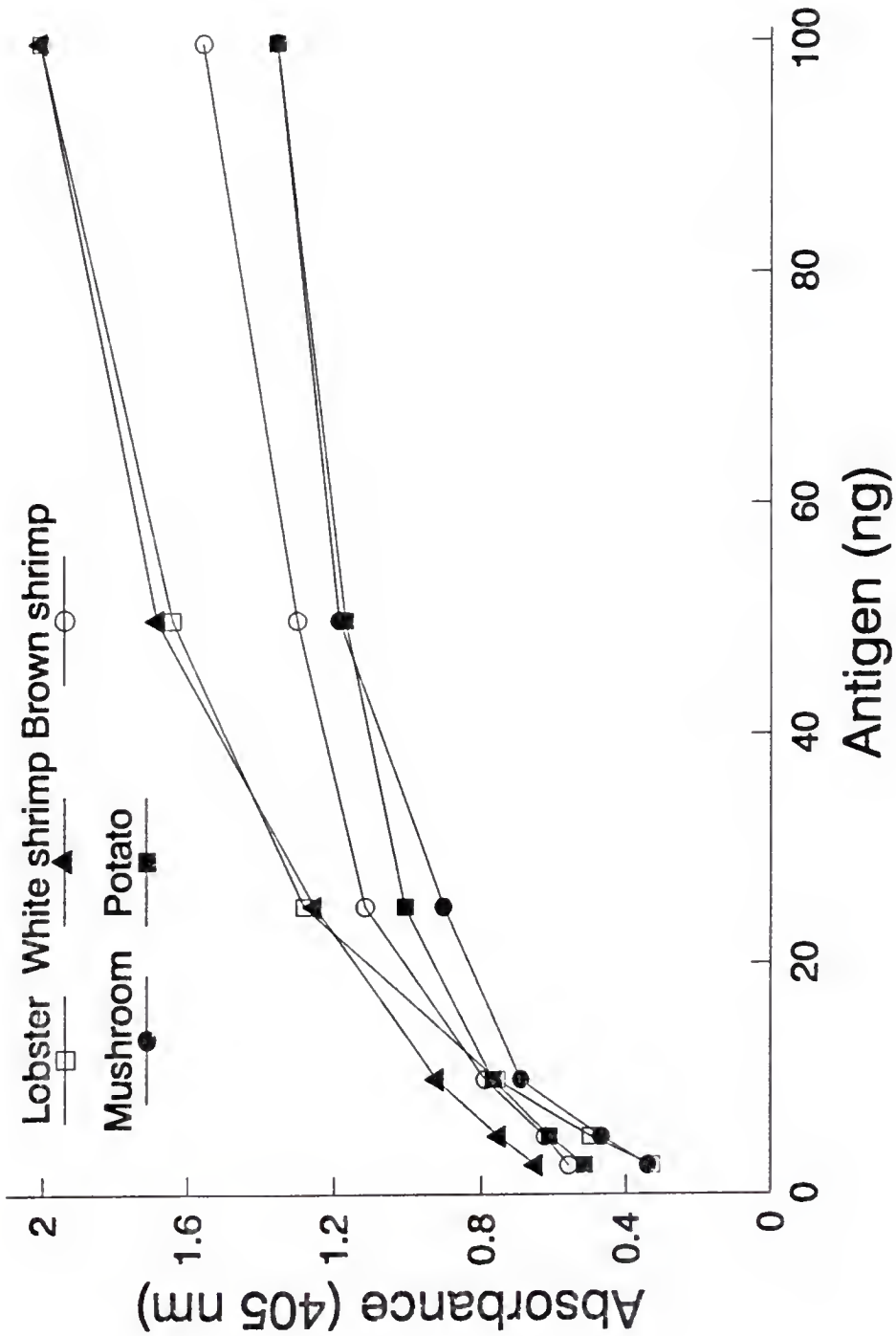

205 kD -
116 kD -
97 kD -
66 kD -
45 kD -
29 kD -
SDS-6H
Anti-lobster
PPO antibody

Figure 13. The Titer Determination of Antibody against Florida Spiny Lobster PPO versus Mushroom, Potato, Florida Spiny Lobster, White Shrimp, and Brown Shrimp PPO; Mushroom, Potato, Lobster, White Shrimp, and Brown Shrimp PPO at 2.5 - 100 ng/well Were Used to React with Anti-lobster PPO Antibody at 2.5 μg/well.


Absorbance (405 nm)
2
1.6
1.2
0.8
0.4
0
0
20
40
60
80
100
Antigen (ng)
Lobster
White shrimp
Brown shrimp
Mushroom
Potato

Immunological Characteristics of PPO

The absorbance at 405 nm of a lobster PPO-antibody reaction mixture in the presence of various competitors (lobster, white shrimp, brown shrimp, mushroom, and potato PPO) was determined by ELISA (Figure 14). The color intensity was reduced by 18 - 79% of the control (Abs. = 0.967) when the various competitor-antibody mixtures containing 12.5 ng competitor and 5 μg antibody per sample well were added to the microplate. The increase of the competitor up to 100 ng/well slightly reduced the color intensity. For mushroom PPO, the competition with lobster PPO for antibody however was not enhanced even when mushroom PPO was added at 100 ng/well. The result again indicated that these PPOs were partially cross-reactive and therefore shared similar structural components. From the extent of competition for lobster PPO-specific antibody, white shrimp and brown shrimp PPO were shown to share more antigenic similarity to lobster PPO than potato and mushroom PPO (in descending order).

Complete transfer of PPO bands along with protein standards from an acrylamide gel onto a nitrocellulose membrane was achieved following electro-transferring; the staining of the treated gels with Coomassie blue revealed no protein bands. The purified lobster, white shrimp, brown shrimp, mushroom, and potato PPO had only one protein band, while crude lobster PPO had 3 isozymes (Figure 15). The nitrocellulose membrane, following staining with lobster PPO-specific antibody, was found to contain dark bands corresponding to potato, lobster, white shrimp, and brown shrimp PPO and the crude lobster PPO preparation. Three dark bands were observed for both the potato PPO and the crude lobster PPO preparation, while only one dark band was found for purified lobster,

Figure 14. Analysis of Antigenic Properties of Purified Mushroom, Potato, Florida Spiny Lobster, White Shrimp, and Brown Shrimp Polyphenol Oxidase (PPO) by Competitive ELISA. Lobster PPO (10 ng/well) as the Antigen Was Plated and Reacted with Anti-lobster PPO Antibody (5 μg/well) in the Presence of 12.5 - 100 ng/well of Mushroom, Potato, Florida Spiny Lobster, White Shrimp, and Brown Shrimp PPO as Competitors.

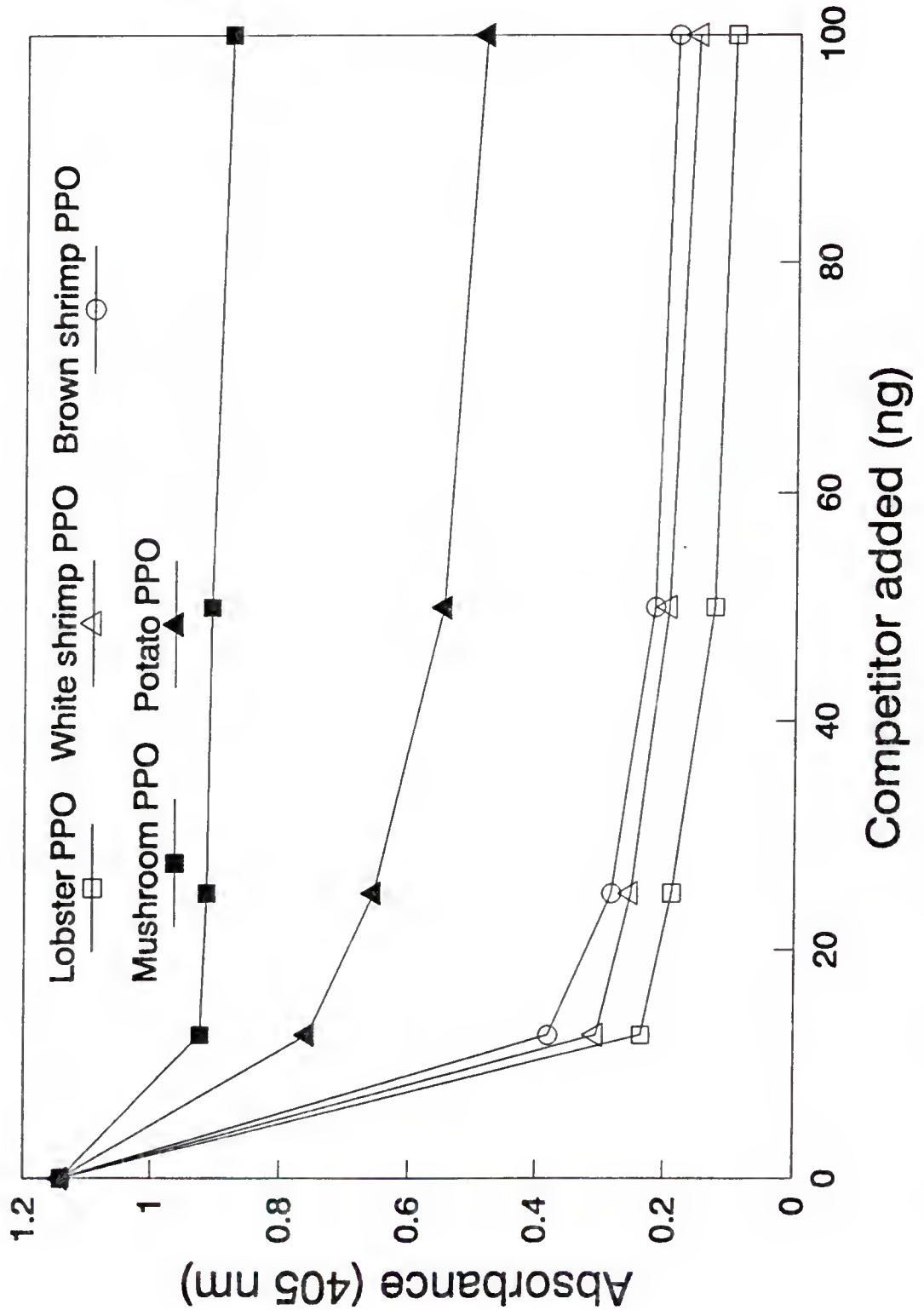

Absorbance (405 nm)
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
Competitor added (ng)
Lobster PPO
White shrimp PPO
Brown shrimp PPO
Mushroom PPO
Potato PPO

Figure 15. SDS-PAGE Profile of Protein Standard (SDS-6H), Purified Mushroom (M), Potato (P), Florida Spiny Lobster (L), White Shrimp (W), and Brown Shrimp (B) PPO, and a Crude Lobster PPO Preparation (CL) under Reduction Condition on a Nitrocellulose Membrane

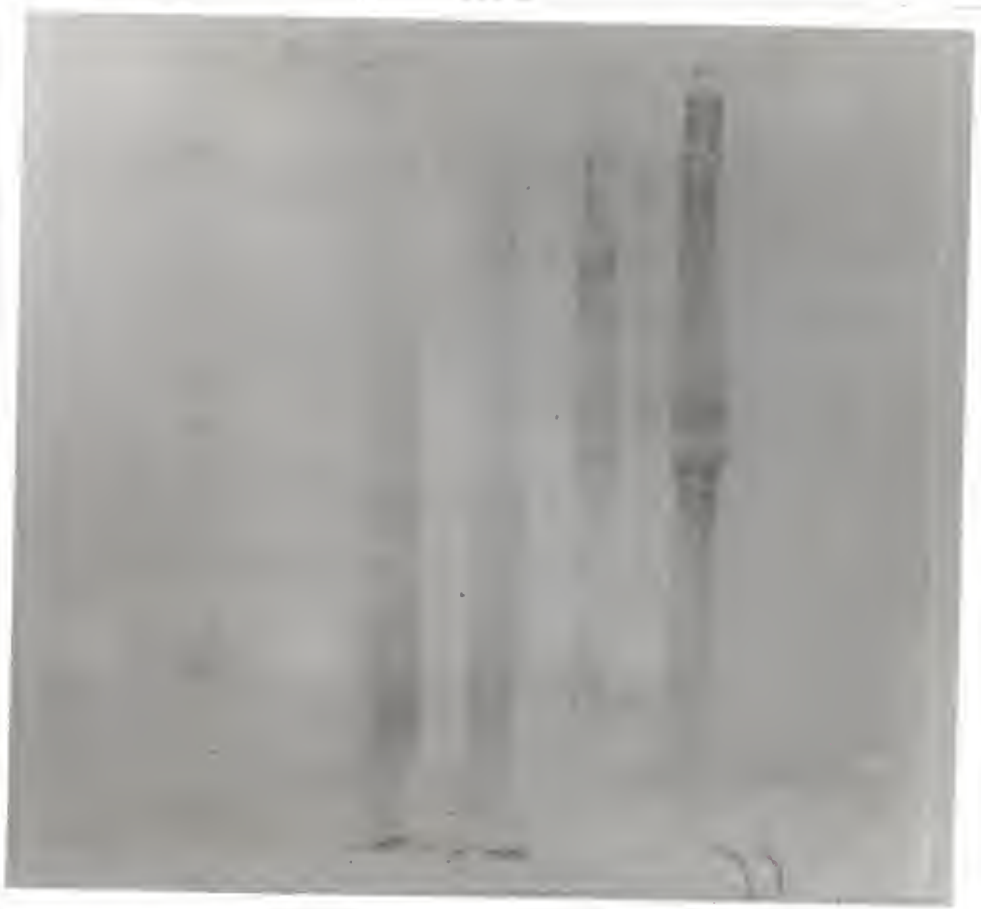
- 205 kD
- 66 kD
- 45 kD
P
M
B
W
L
CL
SDS-6H

white shrimp, and brown shrimp PPO (Figure 16). Incomplete protein transfer or torsional changes in protein structure following electro-transfer process was possibly responsible for the failure of mushroom PPO that not to form a dark band with lobster PPO-antibody. Result from this study did not support the competitive ELISA experiment obtained for mushroom PPO. However, our previous findings that PPO from potato and crustacean sources shared similar structural components was further demonstrated.

Spectropolarimetric Analysis of PPO

Lobster, white shrimp, and brown shrimp PPO had similar circular dichroic spectra (Figures 19, 20, and 21), which were different from those of mushroom and potato (Figures 17 and 18). They all varied in their secondary structures (α-helix, β-sheet, β-turn, and random coil) (Table 3). For example, white shrimp PPO had a higher percentage of α-helix than brown shrimp PPO; they both showed the same broad negative ellipticity between 207 and 220 nm. The percentage of α-helix of mushroom, potato, and crustacean PPOs estimated using the SSE program were close to the values that calculated according to the formula of Greenfield and Fasman (1969). The crustacean PPO, in general, had a higher percentage of α-helix and lower percentage of β-turn than mushroom and potato PPO (Table 3). The percentages of the secondary strucutures of these PPO estimated from the SSE program may not represent the absolute values. However, the results from this study showed that PPO from various sources possessed varied secondary structures, with the crustacean sources producing PPO which showed very similar secondary structure.

Figure 16. Determination of the Specific Reactivity of Anti-lobster PPO Antibody with a Crude Lobster PPO Preparation (CL), Purified Mushroom (M), Potato (P), Florida Spiny Lobster (L), White Shrimp (W), and Brown Shrimp (B) PPO as well as SDS-6H Protein Standards as Analyzed by Immunoblotting Following SDS-PAGE



P M B W L CL SDS-6H

Figure 17. The Far UV (250 - 200 nm) Circular Dichroic Spectra of Mushroom PPO; Four-mL of PPO (10 - 20 μg/mL) in 0.5 mM Sodium Phosphate Buffer (pH 6.5) Was Analyzed at Ambient Temperature.

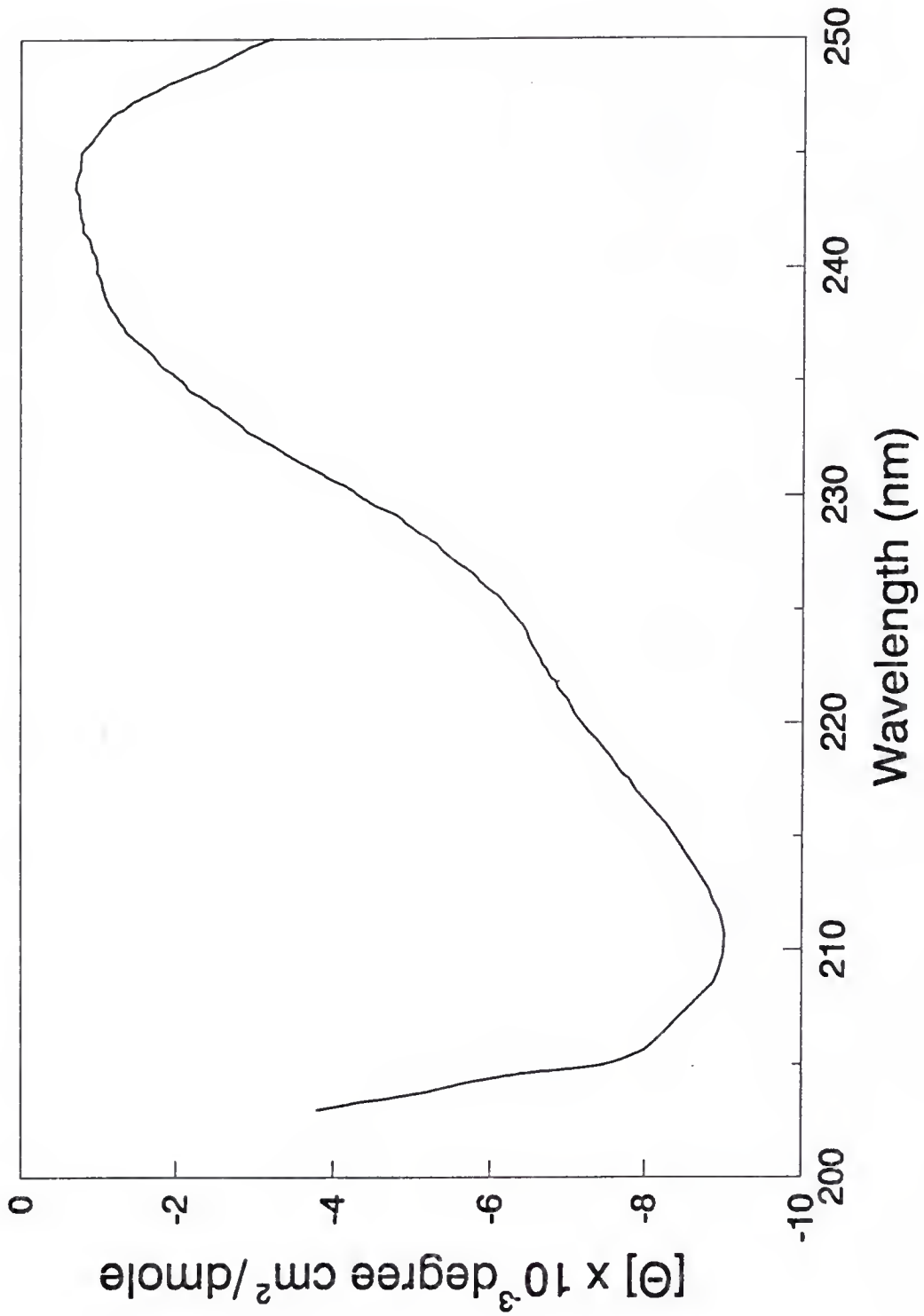

[Θ] x 10^{-3}degree cm^2/dmole
0
-2
-4
-6
-8
-10
200
210
220
230
240
250
Wavelength (nm)

Figure 18. The Far UV (250 - 200 nm) Circular Dichroic Spectra of Potato PPO; Four-mL of PPO (10 - 20 μg/mL) in 0.5 mM Sodium Phosphate Buffer (pH 6.5) Was Analyzed at Ambient Temperature.

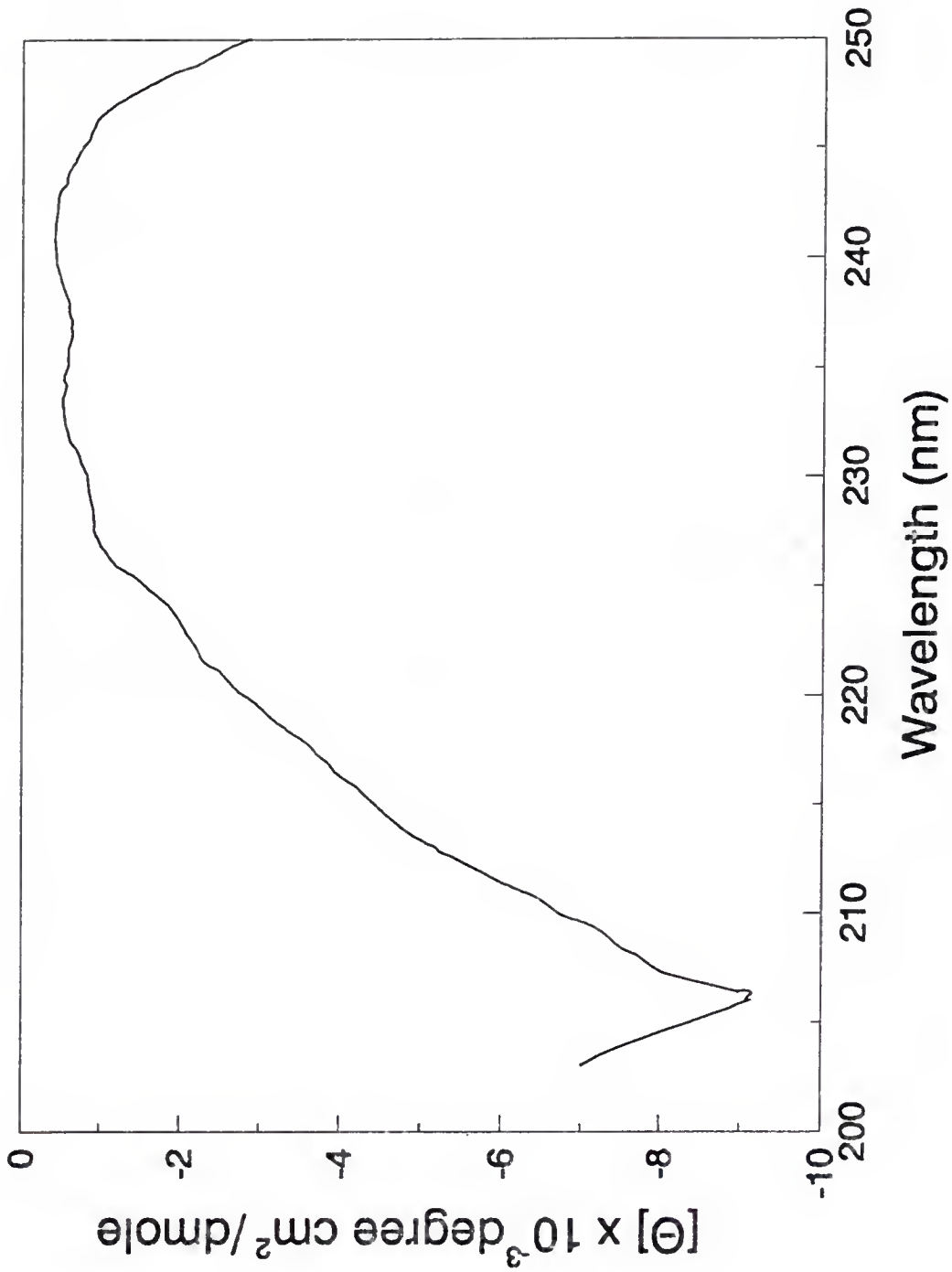

[Θ] x 10^-3 degree cm^2/dmole
0
-2
-4
-6
-8
-10
200
210
220
230
240
250
Wavelength (nm)

Figure 19. The Far UV (250 - 200 nm) Circular Dichroic Spectra of Florida Spiny Lobster PPO; Four-mL of PPO (10 - 20 μg/mL) in 0.5 mM Sodium Phosphate Buffer (pH 6.5) Was Analyzed at Ambient Temperature.

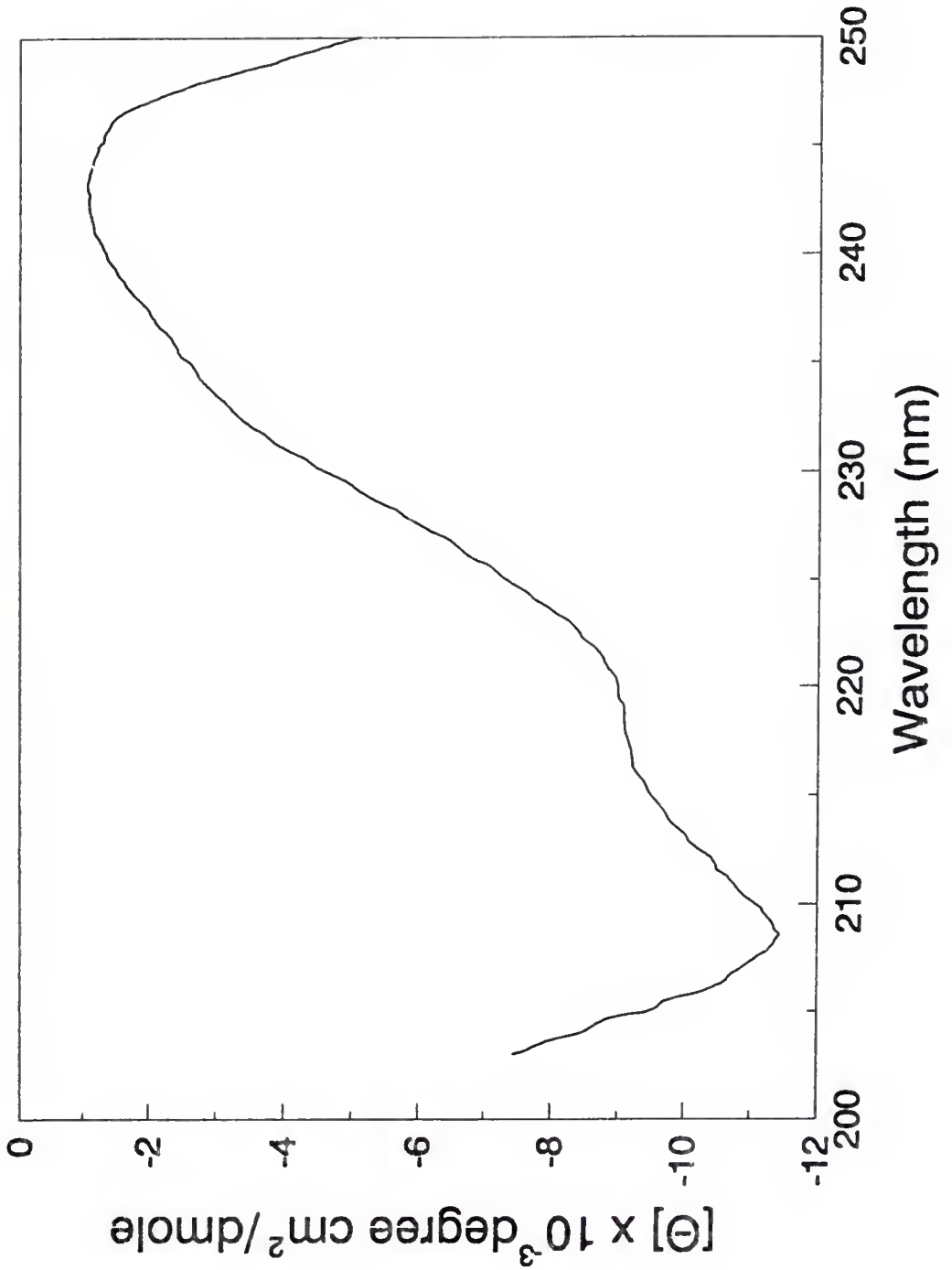

[Θ] x 10^3 degree cm^2/dmole
0
-2
-4
-6
-8
-10
-12
200
210
220
230
240
250
Wavelength (nm)

Figure 20. The Far UV (250 - 200 nm) Circular Dichroic Spectra of White Shrimp PPO; Four-mL of PPO (10 - 20 μg/mL) in 0.5 mM Sodium Phosphate Buffer (pH 6.5) Was Analyzed at Ambient Temperature.

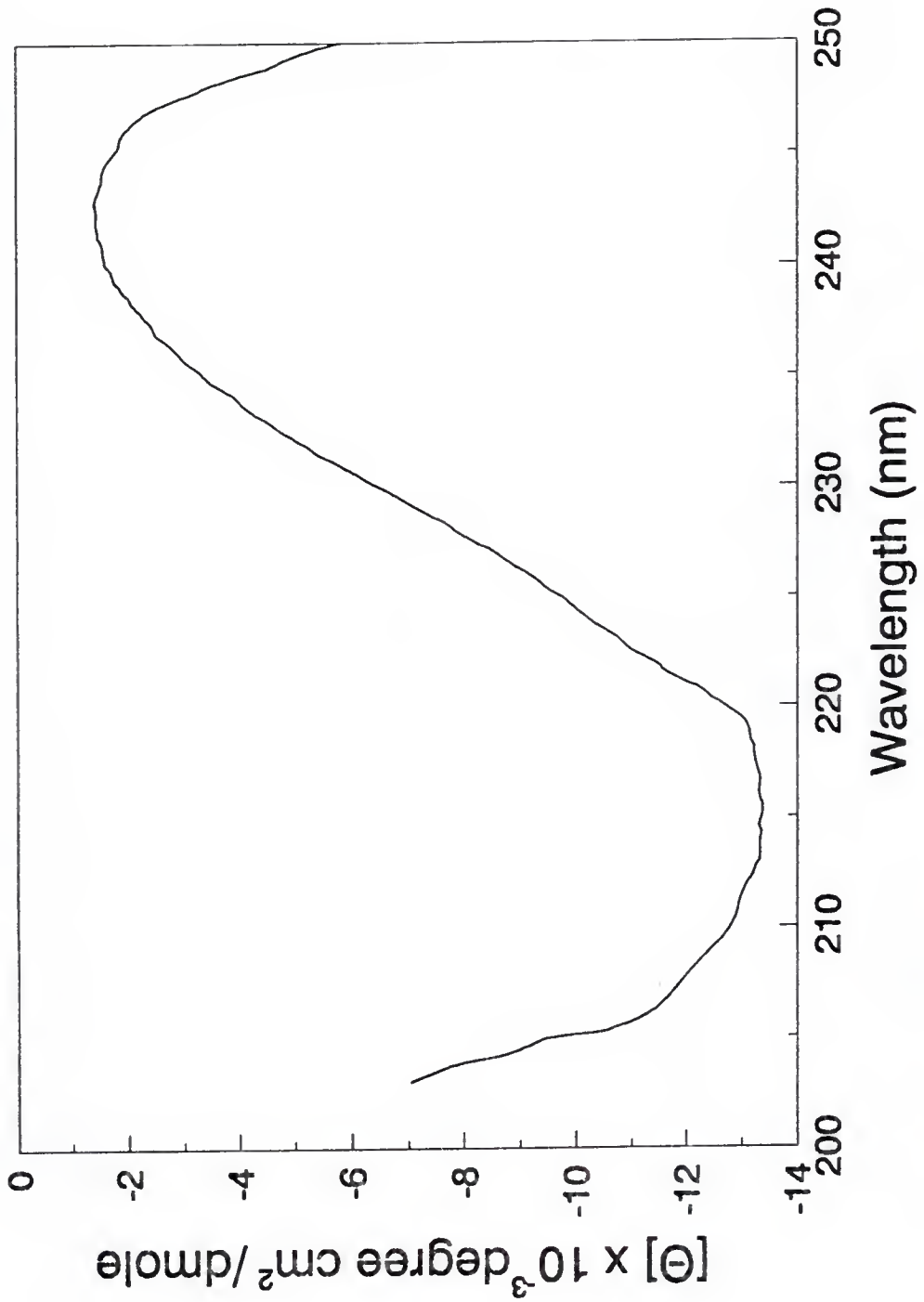

[Θ] x 10^3 degree cm^2/dmole
0
-2
-4
-6
-8
-10
-12
-14
200
210
220
230
240
250
Wavelength (nm)

Figure 21. The Far UV (250 - 200 nm) Circular Dichroic Spectra of Brown Shrimp PPO; Four-mL of PPO (10 - 20 μg/mL) in 0.5 mM Sodium Phosphate Buffer (pH 6.5) Was Analyzed at Ambient Temperature.

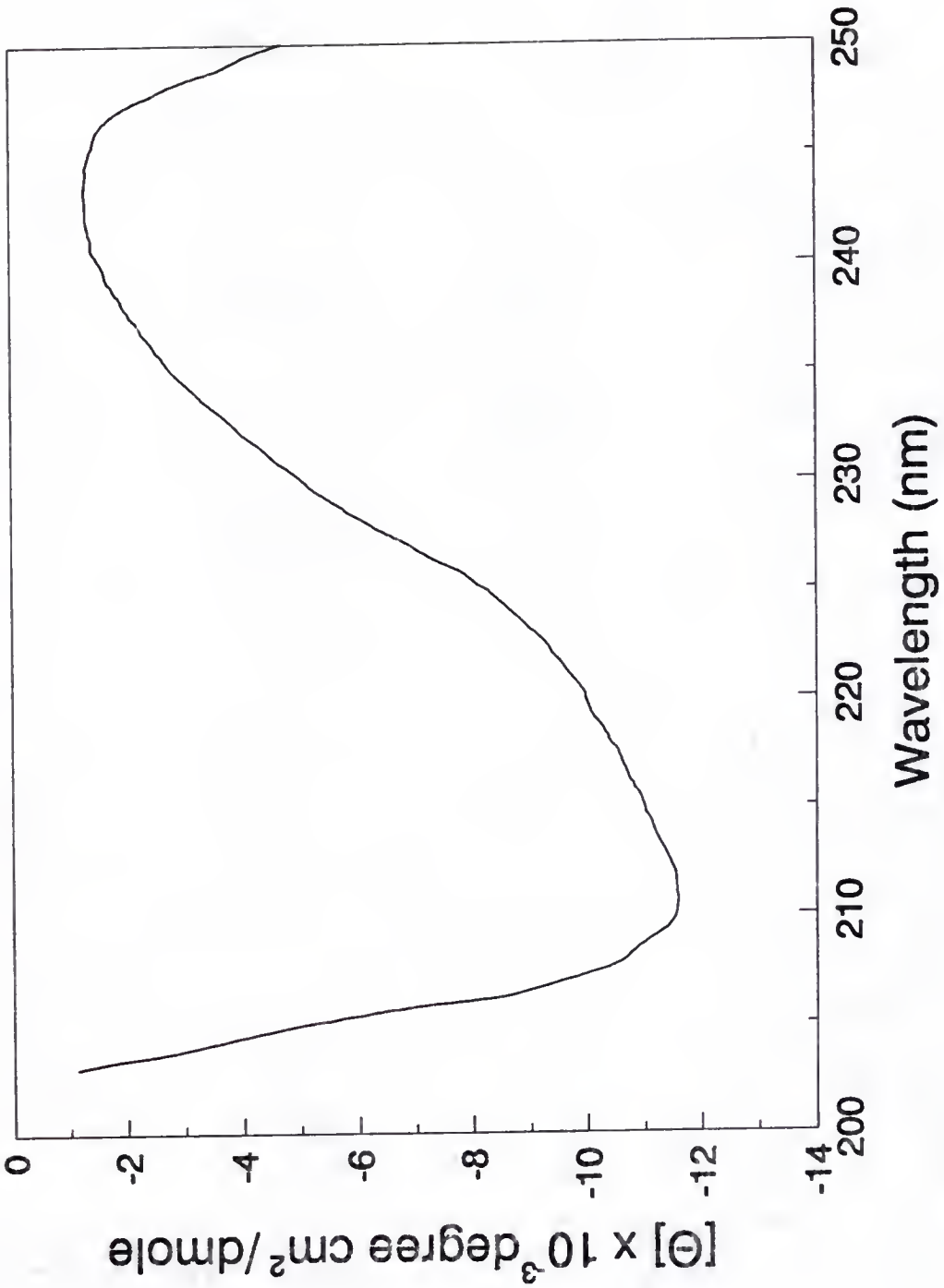

0
-2
-4
-6
-8
-10
-12
-14
200
210
220
230
240
250
Wavelength (nm)
[Θ] x 10^{-3}degree cm^2/dmole

Table 3. Secondary Structure Estimates of Various Polyphenol Oxidases (PPOs) from Far UV Circular Dichroic Spectra

PPO	% of Secondary structure				
	α-helix		β-sheet	β-turn	random coil
Mushroom	18.3	18.1*	17.3	24.8	39.5
Potato	14.8	13.1*	34.6	28.4	22.2
Lobster	24.4	25.1*	26.2	21.4	29.9
White shrimp	29.0	29.7*	30.0	11.3	29.7
Brown shrimp	20.1	20.0*	22.3	15.2	42.4

The circular dichroic spectra of PPO was scanned at the far UV (250 - 200 nm) range. Four-mL PPO (10 - 20 μg/mL) in 0.5 mM sodium phosphate buffer (pH 6.5) was analyzed at ambient temperature

*Calculated according to the formula proposed by Greenfield and Fasman (1969)

Conclusion

Differences in the percentage of secondary structure (α-helix, β-sheet, β-turn, and random coil) among polyphenol oxidases from mushroom, potato, and crustacean were revealed by spectropolarimetric analysis. Using competitive ELISA technique, white shrimp and brown shrimp PPOs were shown to share more antigenic determinants similar to that of lobster PPO than potato and mushroom PPOs when these enzymes were tested against anti-lobster PPO antibody. This result was correlated to the previous finding that crustaceans PPO had higher percentage of α-helix than that of potato and mushroom PPOs.

INHIBITION MECHANISM OF KOJIC ACID ON SOME PLANT AND CRUSTACEAN POLYPHENOL OXIDASES

Introduction

Unfavorable darkening of many food products resulting from enzymatic oxidation of phenols to orthoquinones by polyphenol oxidase (PPO, E.C. 1.14.18.1) has been of great concern to food technologists and processors (Joslyn and Ponting, 1951). The darkening of food products, although innocuous to consumers, causes a decrease in market value and economic loss because it connotes spoilage.

Enzymatic browning of plant products and crustaceans due to PPO activity has been widely studied (Bailey et al., 1960a, 1960b; Ferrer et al., 1989a; Flurkey and Jen, 1978; Harel et al., 1966; Ogawa et al., 1984; Walker, 1962, 1964). Compounds capable of inhibiting melanosis in these products through the interference of PPO mediated reactions or through the reduction of orthoquinones to diphenols have been identified (Bailey and Fieger, 1954; Ferrer et al., 1989b; Golan-Goldhhirsh and Whitaker, 1984; Harel et al., 1967; Madero and Finne, 1982; Palmer and Roberts, 1967; Robb et al., 1966; Sayavedra-Soto and Montgomery, 1986; Wagner and Finne, 1984; Walker, 1975, 1976). However, the number of chemicals that can actually be used in food systems to inhibit melanosis is limited due to off-flavors, off-odors, toxicity, and economic feasibility (Eskin et al., 1971).

Sulfiting agents have been widely used to prevent melanosis in agricultural and seafood products. Due to health concerns, the use of sulfiting agents as food additives has been re-evaluated by FDA and in some products, banned for use (Lecos, 1986). It has become necessary to search for alternatives that show effective inhibition on melanosis but are devoid of health concerns to consumers.

Kojic acid (5-hydroxy-2-hydroxymethyl-γ-pyrone) is a fungal metabolite produced by many species of *Aspergillus* and *Penicillium* (Kinosita and Shikata, 1964; Parrish et al., 1966). It possesses antibacterial and antifungal activity (Morton et al., 1945). Saruno et al. (1979) demonstrated that kojic acid from *Aspergillus albus* inhibited mushroom tyrosinase activity. This compound was also shown to inhibit melanosis in pink shrimp (Applewhite et al., 1990). Kojic acid mixed with ascorbic acid and citric acid constitutes a Japanese product which is used as a tyrosinase inhibitor in foods. Since only limited information was available on the inhibitory effect of kojic acid on PPO, this study was undertaken to investigate the inhibitory activity of this compound on mushroom, plant (potato and apple), and crustacean (white shrimp, Florida spiny lobster, and grass prawn) PPOs and to elaborate on mechanisms involved.

Materials and Methods

Mushroom (*Agaricus bispora*) tyrosinase with an activity of 2,200 units/mg solid was purchased from Sigma Chemical Co. Russet potatoes and Red Delicious apples were purchased from a local supermarket. Fresh Florida spiny lobster (*Panulirus argus*) tails were obtained from Dr. R. A.

Gleeson, Whitney Marine Laboratory, Marineland, FL, and transported in ice to the laboratory. Fresh, nonsulfited white shrimp (*Penaeus setiferus*) was obtained from a local seafood store. Grass prawn (*Penaeus monodon*) frozen in dry ice was provided by Dr. J. S. Yang, Food Industry Research and Development Institute, Hsinchu, Taiwan, Republic of China.

Extraction and Purification of Potato PPO

Potato PPO was extracted following the methods of Patil and Zucker (1965) with slight modification. Potato peel (130 g) was suspended overnight in 3 volumes of cold acetone (w/v, -15°C). Frozen tissue was macerated in a Waring blender with 2 volumes of cold acetone (w/v), and the residue obtained following filtration was homogenized again with cold acetone. After centrifugation at 2,500*g* (4°C) for 20 min, the precipitate was collected and dried at this temperature. Dried powder was suspended and stirred for 10 min in 50 mL of 0.02 M acetate buffer (pH 5.7) and then poured through cheesecloth. The filtrate was centrifuged at 2,500*g* (4°C) for 20 min and the supernatant was treated with 1.5 volumes (v/v) of cold acetone (-15°C). The precipitate was dissolved in 40 mL of 0.02 M acetate buffer (pH 5.7), and then was treated again with an equal volume of cold acetone. The acetone-precipitate was dissolved in 0.01 M acetate buffer (pH 5.7) to make a 0.5% (w/v) solution. After removing brown precipitate by centrifugation, one volume of cold acetone was added to the supernatant. The mixture was centrifuged again at 2,500*g* (4°C) for 20 min and the precipitate was redissolved in 0.01 M acetate buffer (pH 5.7). Ammonium sulfate precipitation between 0 and 60% saturation was obtained by centrifugation at 2,500*g* (4°C) for 20 min. The ammonium sulfate-

precipitate was redissolved in 0.05 M potassium phosphate buffer (pH 7.0) and then dialyzed overnight at 4°C against 2 changes of 4L 0.001 M potassium phosphate buffer (pH 7.0).

DEAE-cellulose Chromatography

Crude potato PPO preparation was loaded onto a DEAE-cellulose (0.95 meq/g, Sigma) column (K 26/40, 40 cm length x 26 mm i.d., Pharmacia) which had been equilibrated with 0.001 M potassium phosphate buffer (pH 7.0). Unbound phenolic compounds and proteins were washed off using 250 mL of 0.001 M phosphate buffer (pH 7.0) at a flow rate of 24 mL/hr for 2 hr. Elution of PPO was performed using a linear concentration (0 - 1.0 M) of NaCl. Four-mL fractions were collected using a fraction collector (Model 2110, Bio-Rad), and protein profile of fractions was determined at 280 nm. Fractions showing PPO activity were pooled and concentrated using an Amicon stirred cell (Model 8050) fitted with a YM 10 membrane filter.

Gel Filtration

Partially purified enzyme preparation after DEAE-cellulose chromatography was loaded onto a Sephadex G-100 gel column (K 26/40, Pharmacia) pre-equilibrated with 0.001 M potassium phosphate buffer (pH 7.0). Elution with 400 mL 0.001 M phosphate buffer (pH 7.0) was then performed at a flow rate of 2.4 mL/hr for 15 hr. Four-mL fractions were collected and protein profile of fractions was determined at 280 nm; fractions showing PPO activity were pooled and concentrated using an Amicon stirred cell. Concentrated samples were dialyzed at 4°C overnight

against 3 changes of 2L of elution buffer. Column chromatography was carried out at 4°C.

Extraction and Purification of Apple PPO

The modified method of Stelzig et al. (1972) was followed to purify apple PPO. Apple peel (150 g) was mixed with liquid nitrogen and ground into fine powder using a Waring blender. Powder (4 g) was homogenized at 4°C in 200 mL of 0.1 M sodium phosphate buffer (pH 6.0) containing 0.3 M sucrose, 0.2% cysteine hydrochloride and 1 mL of 2 x 10^{-2} M 2-mercapto-benzothiazole (MBT) in 95% ethanol, followed by centrifugation at 20,000*g* (4°C) for 20 min. The pellet was washed twice with 50 mL 0.1 M phosphate buffer (pH 6.0) containing 0.2% cysteine-HCl and resuspended in this buffer. This suspension was made to 2% with Triton X-100 (Sigma), and then incubated at 25°C for 15 min. Following centrifugation at 40,000*g* (4°C) for 30 min, supernatant was collected and dialyzed at 4°C overnight against 3 changes of 4L distilled H_2O. The dialysate was extracted with cold (-20°C) *n*-butanol, the aqueous phase was collected and further dialyzed overnight at 4°C against H_2O.

Hydroxylapatite Chromatography

A hydroxylapatite gel (Bio-Gel HT hydroxylapatite, Bio-Rad) suspension (200 mL) was poured into a K 40/26 column. It was then washed with 0.001 M sodium phosphate buffer (pH 7.6). Crude apple PPO was loaded onto the column, and 250 mL of 0.005 - 0.3 M (linear gradient) sodium phosphate buffer (pH 7.6) containing 5% ammonium sulfate was used to desorb the enzyme at a flow rate of 24 mL/hr. Four-mL fractions were

collected and protein profile of fractions were recorded at 280 nm; fractions showing PPO activity were pooled and dialyzed overnight (4°C) against 2L H_2O. Dialysate was further concentrated using an Amicon stirred cell fitted with a YM 10 filter.

Extraction and Purification of Grass Prawn PPO

The method of Rolle et al. (1991) was followed. Heads of grass prawn were frozen in liquid N_2 and then ground to a fine powder in liquid nitrogen using a Waring blender. Powder was suspended in 3 volumes (w/v) of 0.05 M sodium phosphate buffer (pH 7.2) containing 1 M sodium chloride (extraction buffer) and 0.2% (v/v) Brij 35, and stirred at 4°C for 3 hr. Following centrifugation at 23,000*g* at 4°C for 30 min, supernatant was fractionated with ammonium sulfate. Ammonium sulfate precipitating between 0 - 40% saturation was collected by centrifugation at 23,500*g* at 4°C for 30 min. Precipitate was resuspended in 50 mL extraction buffer containing 40% ammonium sulfate. After homogenization using a Dounce manual tissue grinder, the sample was centrifuged at 23,500*g* (4°C) for 20 min. The precipitate was homogenized in extraction buffer and centrifuged as previously described. The resulting precipitate was homogenized in extraction buffer, then subjected to high performance hydrophobic interaction chromatography at 4°C using a preparative Phenyl Sepharose CL-4B (Sigma) column (K 16/40) attached to a Dionex gradient pump (Dionex Corp., Sunnyvale, CA). The column was pre-equilibrated with extraction buffer.

PPO in the column was eluted with a stepwise gradient of elution buffer [100% extraction buffer (9 mL), 50% extraction buffer in water (24

mL), and 10% extraction buffer in water (24 mL)], 50% ethylene glycol (12 mL), and then distilled water (150 mL) at a flow rate of 0.2 mL/min. Four-mL fractions were collected and fractions exhibiting PPO activity were pooled and concentrated via ultrafiltration utilizing a YM 10 filter.

Extraction and Purification of Lobster PPO

The modified procedures of Simpson et al. (1987) were followed. Lobster cuticle was emerged in liquid nitrogen and ground into a fine powder using a Waring blender. It was then extracted with 3 parts (w/v) 0.05 M sodium phosphate buffer (pH 7.2) containing 1 M NaCl and 0.2% Brij 35 at 4°C for 3 hr. Following centrifugation at 8,000*g* at 4°C for 30 min, the supernatant was dialyzed at 4°C against 3 changes of 4L of 0.05 M sodium phosphate buffer (pH 6.5).

PPO was purified using a nondenaturing preparative polyacrylamide gel electrophoresis (PAGE). A one-mL aliquot of crude enzyme extract was applied to each of eight gel tubes (12 cm length x 14 mm i.d.) containing 5% acrylamide/ 0.13% bisacrylamide gel, and ran at a constant current of 10 mA/tube. PPO was visualized using a specific enzyme-substrate staining method (Constantidines and Bedford, 1967) using 10 mM DL-β-3,4-dihydroxyphenylalanine (DL-DOPA) in 0.05 M sodium phosphate buffer (pH 6.5). Gels containing PPO were sectioned and homogenized in 0.05 M phosphate buffer (pH 6.5) utilizing a Dounce tissue grinder. Following filtration through a Whatman No. 4 filter paper, the filtrate was concentrated using an Amicon stirred cell fitted with 10 K filter. The lobster PPO was further purified by subjecting the concentrated filtrate to PAGE at 7.5% acrylamide/ 0.2% bisacrylamide gel.

Extraction and Purification of White Shrimp PPO

The combined methods of Simpson et al. (1987) and Rolle et al. (1991) with slight modification were followed to purify white shrimp PPO. Shrimp cephalothorax was frozen in liquid nitrogen and ground using a Waring blender. Shrimp powder was suspended in 3 volumes (w/v) 0.05 M sodium phosphate buffer (pH 7.2) containing 1 M NaCl (extraction buffer) and 0.2% (v/v) Brij 35, and stirred at 4°C for 3 hr. Following centrifugation at 23,000*g* (4°C) for 30 min, the supernatant was fractionated with ammonium sulfate between 0 - 40% saturation; protein precipitate was collected by centrifugation at 23,500*g* at 4°C for 30 min.

Precipitate collected was dissolved in 0.05 M phosphate buffer (pH 7.2) and dialyzed at 4°C overnight against 3 changes of 4L of 0.05 M phosphate buffer (pH 7.2). The dialyzed PPO was loaded onto a DEAE-cellulose (0.95 meq/g) column (K 26/40) pre-equilibrated with 0.05 M phosphate buffer (pH 7.2). Sixty-mL of 0.05 M sodium phosphate buffer (pH 7.2) was used to desorb unbound phenolic compounds and proteins at 0.2 mL/min. Elution of PPO was performed by a linear gradient (0 - 1.0 M) of NaCl with 300 mL 0.05 M sodium phosphate buffer (pH 7.2). Three-mL fractions were collected and the protein estimated by absorbance at 280 nm. Fractions possessing PPO activity were pooled and concentrated using an Amicon stirred cell fitted with YM 10 filter.

PPO was loaded onto a Sephadex G-100 gel column (K 26/40) pre-equilibrated with 0.05 M sodium phosphate buffer (pH 7.2) and then eluted with 300 mL 0.05 M sodium phosphate buffer (pH 7.2) at 0.15 mL/min. Three-mL fractions were collected and protein was estimated by absorbance at 280 nm; fractions showing PPO activity were pooled and concentrated

using an Amicon stirred cell fitted with YM 10 filter. Concentrated PPO was then dialyzed at 4°C overnight against 3 changes of 2L H_2O.

Protein Quantitation and Enzyme Purity Determination

Protein content of all preparations was quantitated using the Bio-Rad Protein Assay kit with bovine serum albumin as standard. Enzyme purity was examined using a mini gel system (Mini-Protean II Dual Slab Cell) (Bio-Rad, 1985b). Plant and crustacean PPO (20 μg protein/well) was loaded and electrophoresis was carried out at constant voltage (200 V) for 35 min. The purity of enzyme preparations were determined by comparing gels stained with 10 mM DL-DOPA in 0.05 M sodium phosphate buffer (pH 6.5) (Constantidines and Bedford, 1967) and then with a Commassie brilliant blue R-250 solution.

Enzyme Activity Assay

Potato PPO activity was determined at 25°C for 5 min by mixing 2.9 mL 0.97 mM chlorogenic acid in 1 mM potassium phosphate buffer (pH 7.0) with 0.1 mL enzyme. Maximal initial velocity for quinone formation was monitored at 395 nm using a DU-7 spectrophotometer. One unit of PPO activity was defined as an increase in absorbance of 0.001/min at 395 nm and 25°C. Apple PPO activity was measured at 30°C for 5 min by mixing 0.2 mL PPO preparation with 1.8 mL of 0.05 M 4-methylcatechol in 0.1 M sodium phosphate buffer (pH 6.0). Maximal initial velocity for quinone formation was determined at 395 nm and one unit of PPO activity was defined as an increase in absorbance of 0.001/min.

White shrimp PPO activity was carried out at 40°C for 5 min by adding 80 μL of PPO to 1.12 mL 10 mM L-DOPA in 0.05 M sodium phosphate buffer (pH 6.5). Maximal initial velocity for dopachrome formation was determined at 475 nm. One unit of PPO activity was defined as an increase in absorbance of 0.001/min at 40°C. Grass prawn and spiny lobster PPO activities were measured by adding 0.1 mL of enzyme to 1.4 mL 10 mM DL-DOPA in 0.05 M sodium phosphate buffer (pH 6.5). The reaction was monitored at 25°C for 5 min. Maximal initial velocity was determined as (ΔA_{475nm}/min) and one unit of PPO activity was defined as an increase in absorbance of 0.001/min at 25°C.

Effect of Kojic Acid on Enzyme Activity

The method of Saruno et al. (1979) was adopted to study the inhibitory effect of kojic acid on PPO activities. PPO preparation was pre-incubated with sodium acetate, potassium or sodium phosphate buffer, and kojic acid (Sigma) solutions in respective buffer at 37°C for 15 min. Following equilibration to ambient temperature, specific substrate for each system in buffer solution was added to the mixture and the change in absorbance of the reaction product, benzoquinone, was spectrophotometrically monitored for 5 min. For control sample, an equivalent volume of buffer was used to replace kojic acid solution. Percentage inhibition (I) was expressed as $[(T - T^*)/T] \times 100$, where T^* and T were enzyme activities in the presence and absence of kojic acid, respectively (Saruno et al., 1979).

The mushroom PPO system was composed of 0.9 mL of kojic acid (20 - 200 μg/mL), 0.1 mL enzyme (1 mg/mL) in distilled H_2O, and 2.0 mL 0.83 mM

L-tyrosine or 5 mM DL-DOPA in 0.05 M sodium acetate buffer (pH 6.8). The reaction was monitored at 475 nm and 25°C. The potato PPO system contained 0.9 mL of kojic acid (20 - 800 μg/mL), 0.1 mL of enzyme, and 2.0 mL 1.4 mM chlorogenic acid or 5 mM catechol in 1.0 mM sodium phosphate buffer (pH 7.0). The reaction was monitored at 395 nm and 25°C. The apple PPO system contained 1.15 mL of kojic acid (0.02 - 2.0 mg/mL), 0.1 mL enzyme, and 2.0 mL of 1.4 mM chlorogenic acid or 5 mM 4-methylcatechol in 0.1 M sodium phosphate buffer (pH 6.0). The reaction was monitored at 395 nm and 30°C.

White shrimp PPO system contained 0.45 mL of kojic acid (20 - 200 μg/mL), 50 μL of enzyme, and 1.0 mL of 5 mM L-DOPA or catechol in 0.05 M sodium phosphate buffer (pH 6.5). The reaction was monitored at 40°C and at 395 and 475 nm, respectively, for catechol and L-DOPA oxidation. Grass prawn and lobster PPO systems contained 0.9 mL of kojic acid (20 - 150 μg/mL), 0.1 mL of enzyme, and 2.0 mL of 5 mM DL-DOPA or catechol in 0.05 M sodium phosphate buffer (pH 6.5). The reaction was monitored at 25°C and 395 and 475 nm, respectively.

Enzyme Kinetics Study

Michaelis constants, K_m, for the various mushroom, plant, and crustacean PPOs were determined using the Lineweaver-Burk equation (Lineweaver and Burk, 1934). The substrates used for mushroom PPO, potato PPO (10,900 units/mg of protein), and apple PPO (97,400 units/mg of protein) were DL-DOPA (0.30 - 3.33 mM) or L-tyrosine (13.8 - 153 μM) in 0.05 M sodium acetate buffer (pH 6.8), chlorogenic acid (0.60 - 6.67 mM) or catechol (0.90 - 10.0 mM) in 1 mM sodium phosphate buffer (pH 7.0), and 4-Methylcatechol (1.0 - 9.5 mM) or chlorogenic acid (1.0 - 7.0 mM) in

0.1 M sodium phosphate buffer (pH 6.0), respectively. Enzyme activity for each plant PPO was monitored as previously described.

L-DOPA and catechol at 1.5 - 7.0 mM and DL-DOPA and catechol at 1.67 - 9.92 mM in 0.05 M sodium phosphate buffer (pH 6.5) were respectively used as substrate for white shrimp PPO (5,400 units/mg of protein) and grass prawn (900 units/mg of protein) and lobster PPO (7,000 units/mg of protein). The assay for white shrimp PPO was conducted for 5 min at 40°C and at 25°C for grass prawn and lobster PPO. Enzyme activity on L-DOPA or DL-DOPA and catechol was monitored at 475 and 395 nm, respectively.

The inhibitory mechanism of kojic acid on enzyme activities was also investigated. Except for the substitution of 0.5 mL of kojic acid solutions for buffer, substrate concentrations used in the previous study were employed. Kojic acid solutions at 0.28, 0.56, and 1.06 mM were used in the mushroom, white shrimp, grass prawn, and lobster PPO assay systems, while those at 0.56, 1.06, and 1.41 mM were used in the potato and apple PPO systems. Prior to the addition of the substrate, an enzyme-inhibitor mixture was pre-incubated at 37°C for 15 min. The assays for mushroom, potato, grass prawn, and lobster PPO were carried out as previously described. Kinetic parameters of apparent K_m (K_{mapp}) and K_i for enzyme activities were also determined according to the equations of Lineweaver-Burk (1934) and Dixon (1953).

The kinetic constants (K_m, K_{mapp}, and K_i) of potato, apple, and white shrimp PPO with DL-DOPA as the substrate were also determined. Sixty microliters of PPO was added to 940 μL of DL-DOPA in 0.05 M sodium phosphate buffer (pH 6.5). The final concentration of DL-DOPA varied from 1.4 to 8.9 mM. The reaction was monitored at 475 nm and 25°C for 10 min.

The inhibitory effect of kojic acid on the enzyme activity in oxidizing DL-DOPA in these systems was determined by adding 50 μL of kojic acid at 0.56 or 1.12 mM to the cuvette containing PPO and sodium phosphate buffer (pH 6.5). The mixture was pre-incubated at 37°C for 15 min. Following the equilibration to ambient temperature, DL-DOPA was added and the reaction was monitored at 475 nm (25°C) for 10 min. All the assays to determine enzyme activity, the inhibitory effect of kojic acid on the various enzymes, and the enzyme kinetics were conducted at least three times with three different sample preparations.

Effect of Pre-incubation Temperature on PPO Inhibition by Kojic Acid

A reaction mixture containing 950 μL of 0.13 mM kojic acid in 0.05 M sodium phosphate buffer (pH 6.5) and 50 μL mushroom tyrosinase (0.5 mg/mL) or spiny lobster PPO (0.5 mg/mL) was pre-incubated for 15 min in a cuvette at 0°, 25°, or 37°C. After the mixture was equilibrated to ambient temperature, 500 μL 10 mM DL-DOPA in 0.05 M sodium phosphate buffer (pH 6.5) was added and the reaction monitored at 475 nm (25°C) for 5 min. For controls, kojic acid was replaced by phosphate buffer. Percent inhibition (I) was expressed as $[(A - A^*)/A] \times 100$, where A and A* were enzyme activities in the absence and presence of the inhibitor (kojic acid), respectively (Saruno et al., 1979).

Similarly, 50 μL potato PPO was added to a cuvette containing 500 μL 1 mM sodium phosphate buffer (pH 7.0) and 450 μL 0.56 mM kojic acid. After incubation at 0°, 25°, or 37°C for 15 min, and equilibration back to ambient temperature, 500 μL 20 mM catechol in 1 mM phosphate buffer (pH 7.0) was added. The reaction was monitored at 395 nm (25°C) for 5 min.

Percent inhibition was determined as described above. For apple PPO, the reaction was carried out at 30°C in 0.1 M sodium phosphate buffer (pH 6.0).

Effect of Kojic Acid on the Hydroxylation Capability of PPO

A 100 μL kojic acid solution (0.35 - 5.63 mM) in 0.05 M sodium phosphate buffer (pH 6.5) with 60 μL mushroom PPO (0.5 mg/mL) in water at ambient temperature for 15 min, 840 μL of 1 mM L-tyrosine in the same buffer was added. The reaction was monitored at 475 nm (25°C) for 90 min. For control sample, kojic acid was replaced with buffer.

Effect of Kojic Acid on O_2 Uptake by PPO Reaction

The effect of kojic acid on the inhibition of PPO was also conducted using a polarographic method. A 0.1 mL aliquot of apple PPO was added to 0.1 mL kojic acid solution (0.28, 0.56, or 1.06 mM) in 0.1 M sodium phosphate buffer (pH 6.0) into the sample chamber of a biological oxygen monitor (YSI model 53, Yellow Springs Instrument Co., Yellow Springs, OH). Following incubation at ambient temperature for 30 min, 2.9 mL of 0.1 M 4-methylcatechol or chlorogenic acid in 0.1 M sodium phosphate buffer (pH 6.0) was added. The reaction was allowed to proceed at ambient temperature for 10 min and the consumption rate of O_2 was monitored using a Brinkmann Servogor 210 recorder at a chart speed of 1 cm/min. The rate of O_2 consumption was determined as the initial slope of the curve; the percent change in O_2 was measured against time. For the control sample, phosphate buffer was used in place of kojic acid. Background O_2

consumption for kojic acid, substrate, and a mixture of the two was also carried out.

A similar study was conducted using lobster PPO; in which 10 mM DL-DOPA or catechol in 0.05 M sodium phosphate buffer (pH 6.5) was used as the substrate. Percent inhibition (I) on the rate of O_2 consumption was defined as $[(U - U^*)/U] \times 100$, where U and U^* were the rate of O_2 consumption in the absence or presence of kojic acid.

Effect of Kojic Acid on Reduction of Cu^{2+}

The method of Andrawis and Kahn (1990) with slight modification was followed. Ten minutes after incubating at ambient temperature, a mixture containing 1 mL kojic acid in 0.05 M sodium phosphate buffer (pH 7.0) and 0.5 mL 0.4 mM cupric sulfate (Fisher) in the same buffer was added to a 0.5 mL aliquot of 4 mM aqueous bathocuproine disulfonic acid. The final concentration of kojic acid in the mixture was between 0.02 to 1.40 mM. After the mixture was incubated at ambient temperature for another 20 min, the absorbance at 483 nm was determined using a DU-40 spectrophotometer (Beckman). For the control sample, 1 mL phosphate buffer was substituted for kojic acid.

Effect of Kojic Acid on Quinone Products

A reaction mixture containing 1.2 mL 10 mM DL-DOPA and 0.8 mL mushroom PPO (0.125 mg/mL) in 0.05 M sodium phosphate buffer (pH 6.5) was incubated at ambient temperature for 30 min. Following red color development due to dopaquinone formation, the mixture was scanned from 220 to 700 nm using a spectrophotometer. The effect of kojic acid on

dopaquinone was also studied by adding 0.6 mL 5.63 mM kojic acid in 0.05 M sodium phosphate buffer (pH 6.5) to a mixture containing 0.2 mL mushroom PPO (0.5 mg/mL) and 1.2 mL 10 mM DL-DOPA. Following incubation at ambient temperature for 30 min, the solution was scanned as previously described. An similar study was also conducted on spiny lobster PPO.

The effect of kojic acid on dopaquinone formation was further investigated using thin-layer chromatography. One-mL mushroom PPO (0.5 mg/mL) was mixed with 1.0 mL 5 mM DL-DOPA in 0.05 M sodium phosphate buffer (pH 6.5) and the reaction was allowed to proceed at ambient temperature for 2 hr. Following the reaction, 0.5 mL kojic acid (1 mg/mL) in 0.05 M sodium phosphate buffer (pH 6.5) was added to 0.5 mL aliquot of the reaction mixture and incubated at ambient temperature. After 2 hr, 80-μL aliquots were spotted on TLC plates (20 x 20 cm Redi/plt Sil, Gel G, Fisher) and the plates developed using a butanol-acetic acid-water (4: 1: 5, v/v) solvent system. An equivalent volume of 10 mM DL-DOPA in 0.05 M sodium phosphate buffer (pH 6.5) was used as standard. The chromatographic pattern was examined and the R_f value for each compound was determined after spraying the plate with a ninhydrin reagent (Sigma). For control sample, an equivalent volume of phosphate buffer was in place of kojic acid.

Enzymatic Activities of Kojic Acid-treated PPO

Two-mL of mushroom, white shrimp, and lobster PPO was individually incubated at ambient temperature for 30 min with 0.5 mL kojic acid (0.56 or 1.12 mM) in 0.05 M sodium phosphate buffer (pH 6.5) in a Spectra/Por Membrane (Spectrum Medical Industries Inc., Los Angels, CA) with molecular

weight cutoff of 12,000 - 14,000. The chamber containing the enzyme-kojic acid mixture was then dialyzed overnight (4°C) against 3 changes of 2 L phosphate buffer. For control sample, kojic acid was replaced with an equivalent volume of buffer. Studies on potato and apple PPO were similarly conducted except kojic acid at 1.12 or 5.60 mM was used. The enzyme activity of mushroom, white shrimp, and lobster PPO was determined by adding 60 μL PPO to the cuvette containing 840 μL of 10 mM DL-DOPA in 0.05 M sodium phosphate buffer (pH 6.5). The reaction was monitored for 5 min at 475 nm and 25°C for mushroom and lobster, and 40°C for white shrimp PPO. For potato and apple PPO, the activity was determined by adding 60 μL enzyme to the cuvette containing 840 μL of 10 mM catechol in 1 mM sodium phosphate buffer (pH 7.0) or 0.1 M sodium phosphate buffer (pH 6.0), respectively. The reaction was monitored for 5 min at 395 nm and 25°C for potato PPO, and 30°C for apple PPO.

Kojic acid residual in enzyme preparations was quantitated using the method of Bentley (1957) with slight modification. Five-hundred μL enzyme preparation was incubated with 1.5 mL of 1% (w/v) $FeCl_3$ solution at ambient temperature for 40 min and absorbance measured at 505 nm. Kojic acid at various concentrations (10-250 μg/mL) was used as standard.

Statistical Analysis

Statistical analysis was carried out using a PC SAS package (SAS, 1985). Duncan's multiple range test was performed at a level of $\alpha = 0.05$ to determine any significant difference among treatments. Unless otherwise specified, all experiments were carried out twice in triplicates.

Results and Discussion

Effect of Kojic Acid on Mushroom PPO Activity

Kojic acid showed a concentration-dependent inhibitory effect on mushroom tyrosinase oxidation of DL-DOPA (Figure 22). Addition of kojic acid (20 μg/mL) to the assay system containing DL-DOPA and L-tyrosine inhibited tyrosinase by 72 and 64%, respectively (Figure 23). This inhibitory effect was elevated to 90% when kojic acid at 80 μg/mL was added. Saruno et al. (1979) reported only 20 to 30% mushroom PPO inhibition when kojic acid at 20 μg/mL was used. The use of different enzyme preparations in these two studies could have affected the inhibitory effectiveness of kojic acid.

Effect of Kojic Acid on Potato PPO Activity

Potato PPO was not as effectively inhibited by kojic acid as mushroom tyrosinase especially when catechol was used as substrate (Figure 24). Approximately 95% of mushroom tyrosinase activity was suppressed when kojic acid at 200 μg/mL was used (Figure 23). However, only 55 and 25% inhibition of potato PPO was achieved for chlorogenic acid and catechol, respectively, under the same conditions. As kojic acid concentration was increased to 800 μg/mL, the inhibition of potato PPO on the oxidation of chlorogenic acid approached 90%. However, the inhibition was only 53% for the oxidation of catechol. Abukharma and Woolhouse (1966) demonstrated that 0.1 mM potassium cyanide or sodium azide and 1 mM 3,4-dichlorophenyl serine gave about 60% inhibition on potato PPO when chlorogenic acid was used as substrate.

Figure 22. Effect of Concentration-related Inhibitory Effect of Kojic Acid on Mushroom Tyrosinase (PPO) Activity on DL-β-3,4-dihydroxyphenylalanine (DL-DOPA); A 0.9 mL Aliquot of Kojic Acid Solution at 20 - 150 μg/mL Was Added to the Assay System.

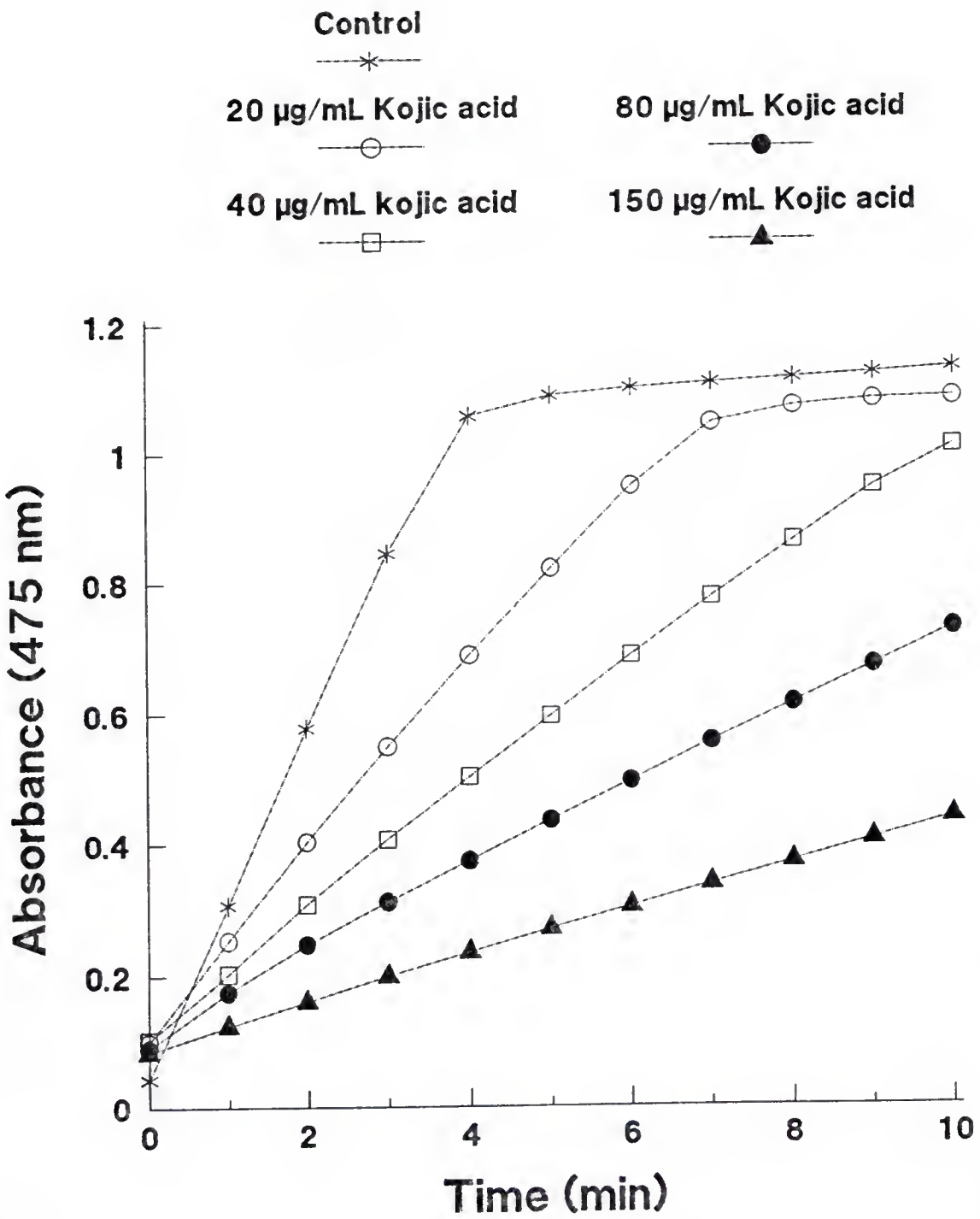

Control
20 µg/mL Kojic acid
80 µg/mL Kojic acid
40 µg/mL kojic acid
150 µg/mL Kojic acid
Absorbance (475 nm)
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
Time (min)

Figure 23. The Concentration-related Inhibitory Effect of Kojic Acid on the Oxidation of DL-DOPA (□) and L-Tyrosine (●) by Mushroom PPO; A 0.9-mL Aliquot of Kojic Acid at 20 - 200 μg/mL Was Added to the Assay Mixture.

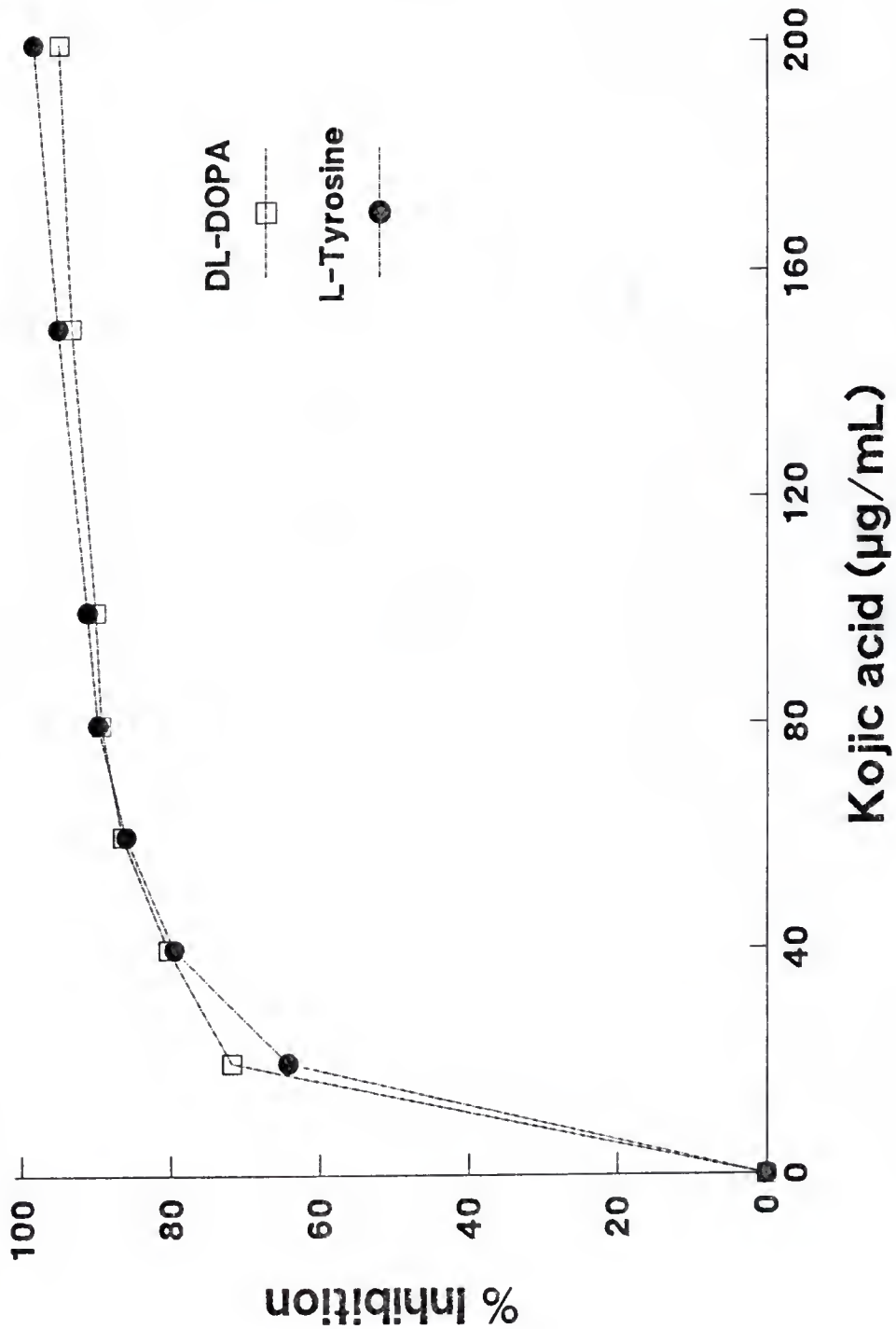
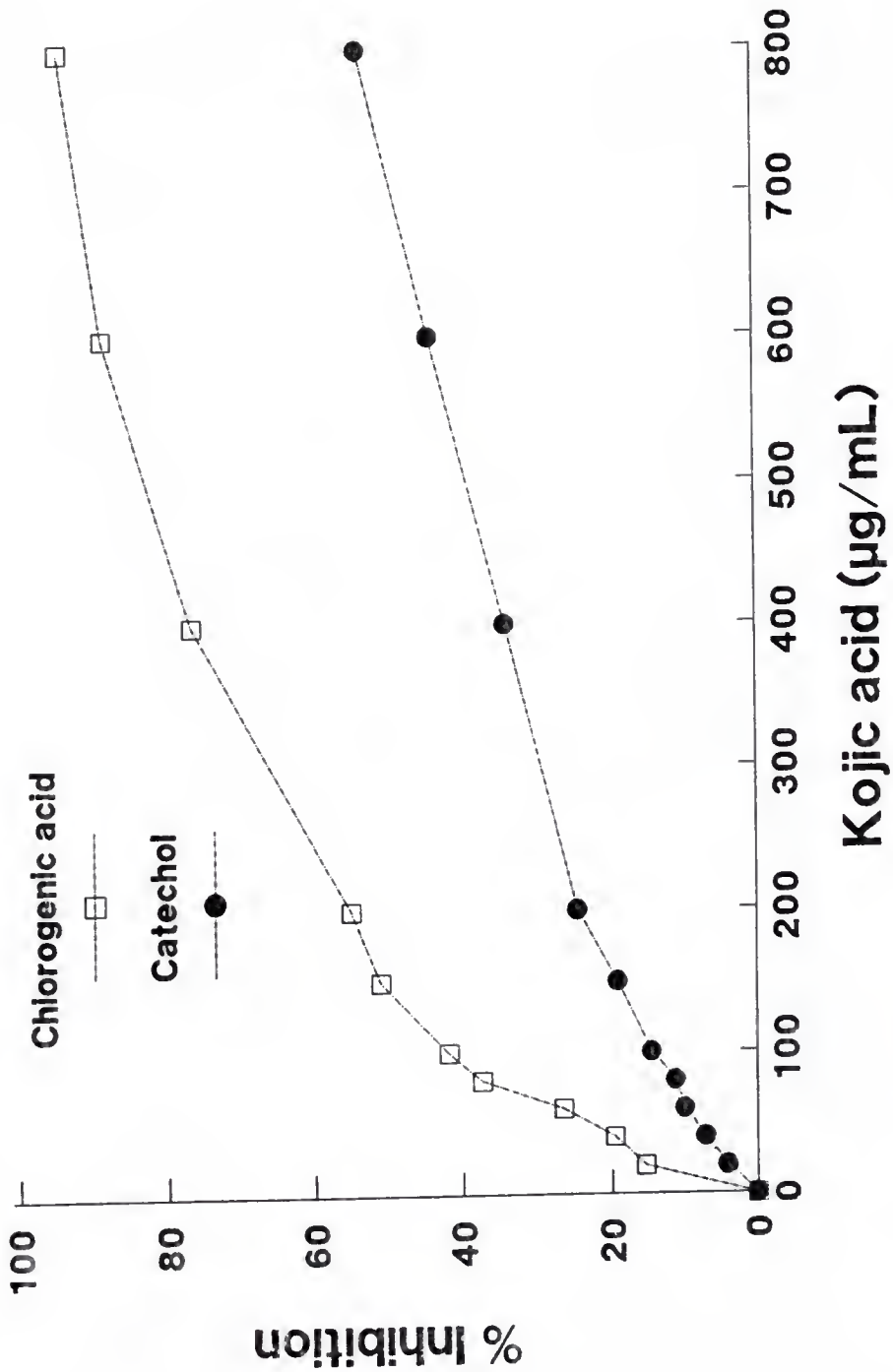

% Inhibition
100
80
60
40
20
0
0
40
80
120
160
200
Kojic acid (µg/mL)
DL-DOPA
L-Tyrosine

Figure 24. The Concentration-related Inhibitory Effect of Kojic Acid on the Oxidation of Chlorogenic Acid (□) and Catechol (●) by Potato PPO; A 0.9-mL Aliquot of Kojic Acid at 20 - 800 μg/mL Was Added to the Assay Mixture.

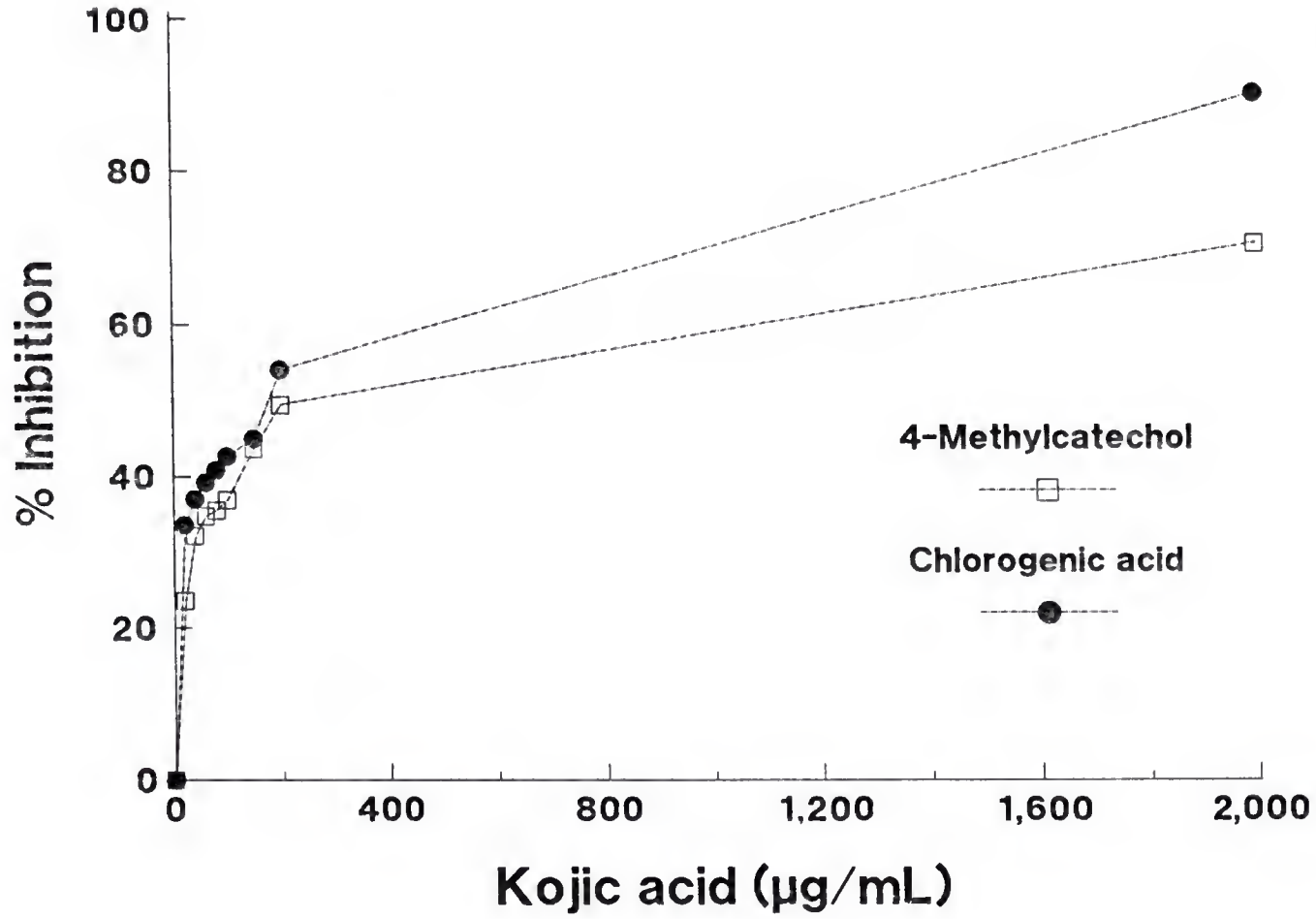

% Inhibition
0
20
40
60
80
100
0
100
200
300
400
500
600
700
800
Kojic acid (µg/mL)
Chlorogenic acid
Catechol

Cysteine and other sulfhydryl-related compounds have been studied for their inhibitory effect on enzymatic browning (Lerner, 1953). Roston (1960) proposed that cysteine inhibits enzymatic blackening caused by the oxidation of L-tyrosine and DL-DOPA by forming addition products with quinones. Muneta and Walradt (1968) studied potato PPO and found that the concentration of cysteine required for comparable inhibition was 5-fold higher with cysteine than for sulfite. These authors also noted that cysteine did not inhibit chlorogenic acid oxidation; and the oxidation of tyrosine to DOPA is more sensitive to cysteine inhibition than the oxidation of DOPA to dopaquinone. Both Muneta and Walradt (1968) and Henze (1956) proposed that cysteine inhibited enzymatic browning by combining with the quinone. The sulfiting agents have been postulated toreact with the enzyme or the quinones to exert the inhibitory effect (Joslyn and Ponting, 1951; Joslyn and Braverman, 1954). Although sulfite agents can effectively inhibit enzymatic browning, they affect the nutritive value either by preserving ascorbic acid or destroying thiamine (Mapson and Wager, 1961; Markakis and Embs, 1966).

Effect of Kojic Acid on Apple PPO Activity

Oxidation rates of 4-methylcatechol and chlorogenic acid by apple PPO decreased as kojic acid concentration increased. Only 50% inhibition occurred for kojic acid at 200 μg/mL (Figure 25). Kojic acid inhibition of apple PPO was similar to potato PPO (Figure 24), but was far less pronounced than for mushroom PPO (Figure 23). Harel et al. (1965) demonstrated that 2,3-naphthalenediol at 5 mM and N-vinyl-2-pyrrolidone at 2.5% respectively inhibited 64 and 60% PPO prepared from apple

Figure 25. The Concentration-related Inhibitory Effect of Kojic Acid on the Oxidation of 4-Methylcatechol (□) and Chlorogenic Acid (●) by Apple PPO; A 1.15-mL Aliquot of Kojic Acid at 0.02 - 2.0 mg/mL Was Added to the Assay Mixture.


100
80
60
40
20
0
% Inhibition
0
400
800
1,200
1,600
2,000
Kojic acid (μg/mL)
4-Methylcatechol
Chlorogenic acid

chloroplasts. Walker (1964) demonstrated that diethylthiocarbamate or dimercaptopropanol at 1 mM was able to inhibit 90% of the oxidation of chlorogenic acid.

Effect of Kojic Acid on Crustacean PPO Activity

Kojic acid inhibition on white shrimp (Figure 26) and grass prawn PPO (Figure 27) was less effective than on spiny lobster (Figure 28). The former two enzymes were only inhibited by about 20%, while the oxidation of DL-DOPA and catechol by lobster PPO was inhibited to 80 and 70%, respectively, when kojic acid at 20 μg/mL was used. Kojic acid appeared to be more effective in inhibiting lobster PPO than white shrimp and grass prawn PPO and plant PPO. L-Tyrosine, L-cysteine, and sodium diethyl dithiocarbamate effectively inhibited the oxidation of DOPA (Antony and Nair, 1975). *p*-Aminobenzoic acid (PABA) and NaN_3 was more effective than EDTA and cysteine in inhibiting pink and white shrimp PPO (Simpson et al., 1988a). On the basis of the molar ratio between crustacean PPO and inhibitor used, kojic acid was found to be less effective in inhibiting white shrimp, grass prawn, and lobster PPO than PABA and NaN_3 on pink and white shrimp PPO.

Enzyme Kinetics

Kinetic parameters (K_m, K_{mapp}, and K_i) for the various PPOs utilizing different substrates in the absence or presence of kojic acid, and the type of inhibition are listed in Table 4. Kojic acid was a competitive

Figure 26. The Concentration-related Inhibitory Effect of Kojic Acid on the Oxidation of L-DOPA (□) and Catechol (●) by White Shrimp PPO; A 0.25-mL Aliquot of Kojic Acid (20 - 200 μg/mL) Was Added to the Assay Mixture.

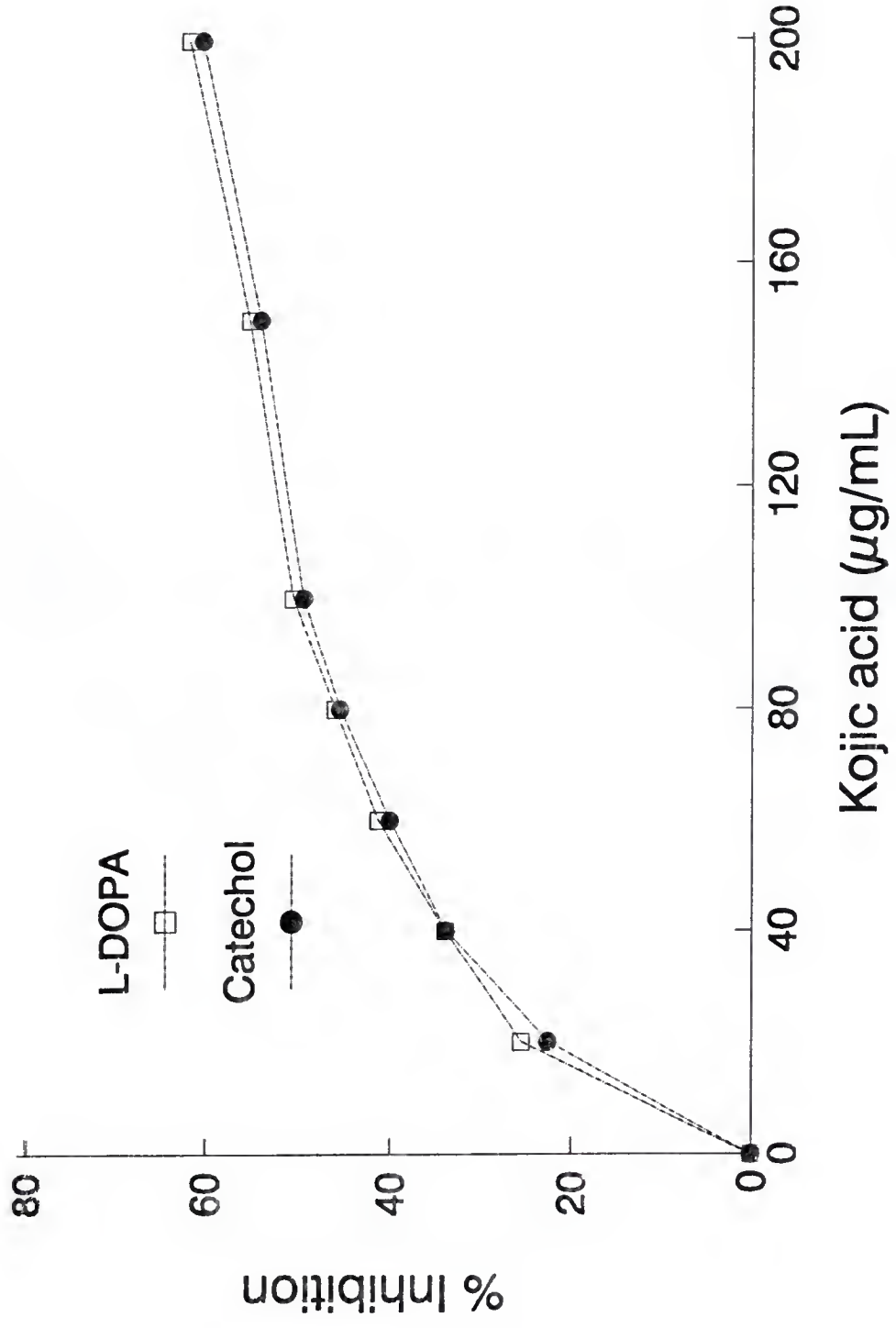

% Inhibition
0
20
40
60
80
0
40
80
120
160
200
Kojic acid (μg/mL)
L-DOPA
Catechol

Figure 27. The Concentration-related Inhibitory Effect of Kojic Acid on the Oxidation of DL-DOPA (□) and Catechol (●) by Grass Prawn PPO; A 0.9-mL Aliquot Kojic Acid (20 - 150 μg/mL) Was Added to the Assay Mixture.

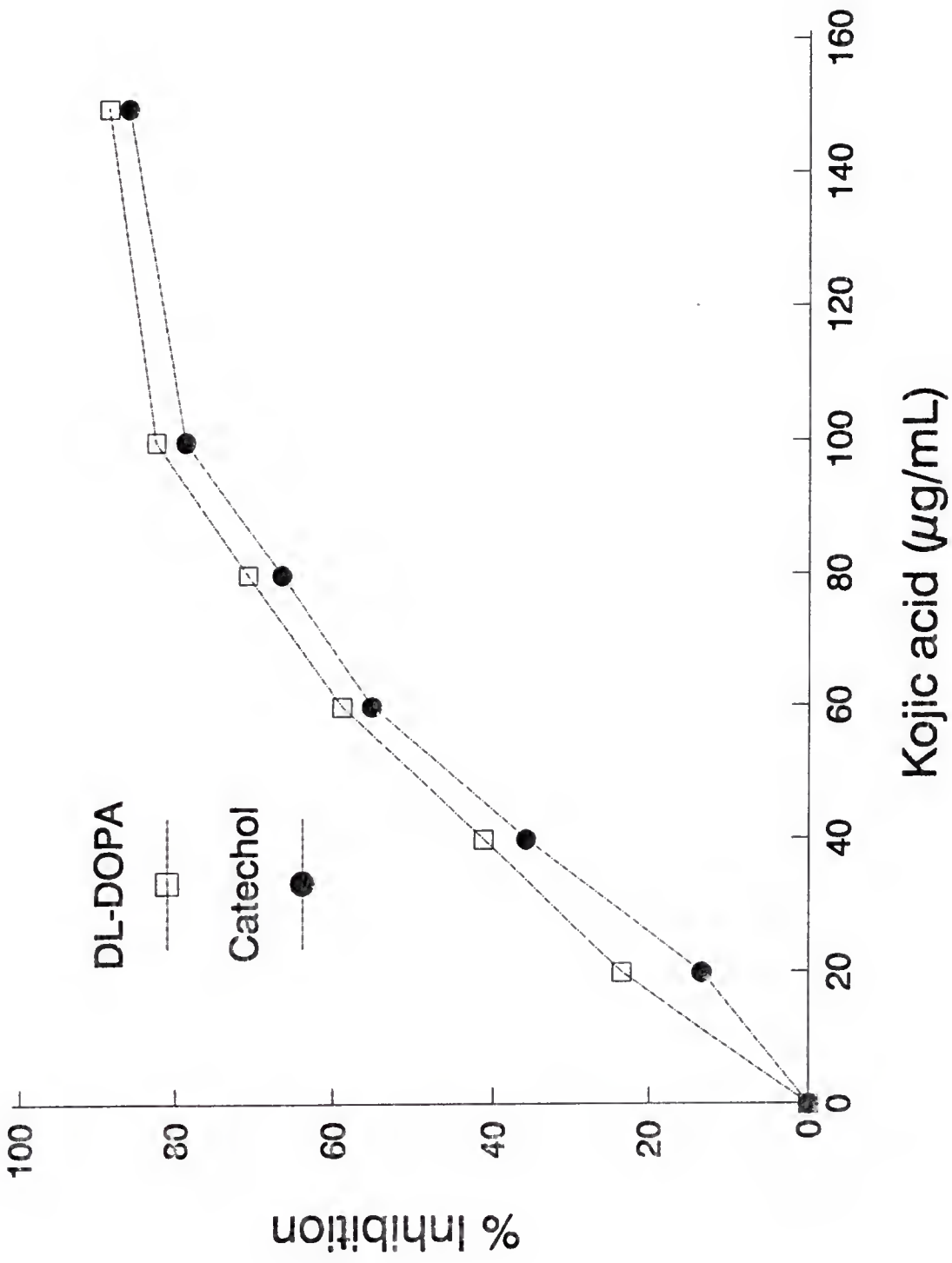

% Inhibition
100
80
60
40
20
0
0
20
40
60
80
100
120
140
160
Kojic acid (μg/mL)
DL-DOPA
Catechol

Figure 28. The Concentration-related Inhibitory Effect of Kojic Acid on the Oxidation of DL-DOPA (□) and Catechol (●) by Lobster PPO; A 0.9-mL Aliquot Kojic Acid (20 - 150 μg/mL) Was Added to the Assay Mixture.

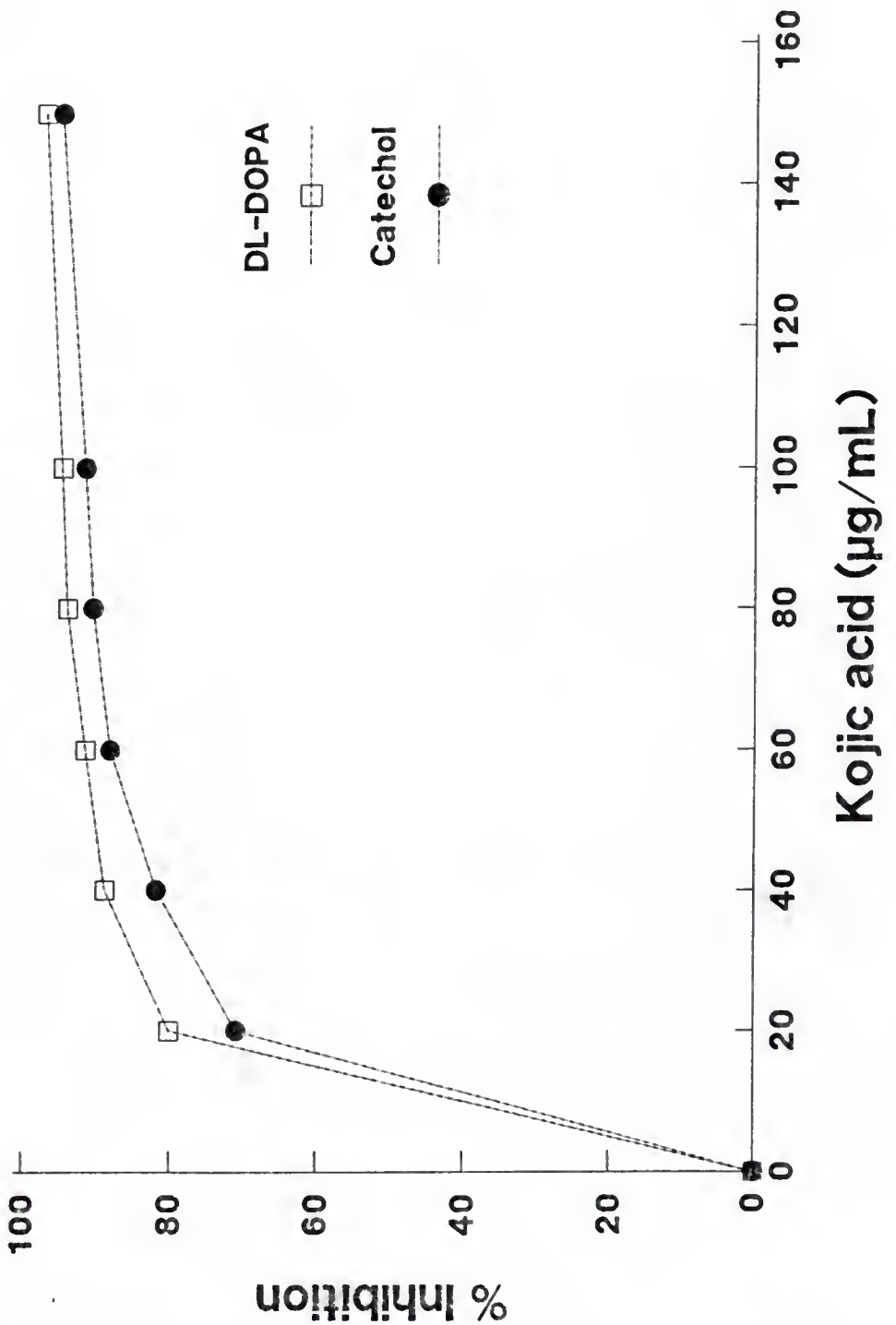

% Inhibition
100
80
60
40
20
0
0
20
40
60
80
100
120
140
160
Kojic acid (µg/mL)
DL-DOPA
Catechol

Table 4. Inhibitory Mechanism of Kojic Acid on Polyphenol Oxidase Obtained from Various Sources

Enzyme source	Substrate	Michaelis constant (K_m), mM	Type of inhibition	Apparent K_m, mM	Inhibitor constant (K_i), mM
Mushroom	L-tyrosine	0.69	Competitive	2.02	0.03
	DL-DOPA	0.24	Mixed	0.66	0.02
Potato	Chlorogenic acid	5.20	Competitive	7.23	0.60
	Catechol	7.89	Competitive	10.5	0.71
	DL-DOPA	0.06	Competitive	0.08	0.06
Apple	4-methylcatechol	3.85	Competitive	4.28	0.13
	Chlorogenic acid	8.20	Competitive	10.3	0.06
	DL-DOPA	0.04	Competitive	0.07	0.03
White Shrimp	L-DOPA	3.48	Mixed	4.29	0.15
	Catechol	4.27	Mixed	5.18	0.18
	DL-DOPA	3.20	Mixed	4.20	0.09
Spiny lobster	DL-DOPA	3.27	Mixed	4.37	0.07
	Catechol	4.98	Mixed	7.31	0.10
Grass prawn	DL-DOPA	3.64	Mixed	7.77	0.05
	Catechol	5.29	Mixed	7.78	0.07

inhibitor for both potato and apple PPO, but a mixed-type inhibitor for white shrimp, grass prawn and Florida spiny lobster PPO. For mushroom PPO, it was a competitive inhibitor for L-tyrosine, while a mixed-type inhibitor for DL-DOPA.

The Michaelis constant for the oxidation of DL-DOPA and L-tyrosine by mushroom tyrosinase was 0.29 mM and 0.69 mM, respectively. The K_m value for DL-DOPA was close to that for the oxidation of catechol (0.22 mM) and chlorogenic acid (0.22 mM) reported by Sisler and Evans (1958) but was lower than the values reported by Smith and Kruger (1962) on catechol (2.5 - 4.0 mM). Similarly, the Michaelis constant for L-tyrosine was lower than that for the oxidation of *p*-cresol (1.5 - 10 mM) (Sisler and Evans, 1958). Mayer et al. (1966) have attributed this discrepancy to different enzyme preparations and assay methods used. Based on the K_m values for these two substrates, it was noted that mushroom tyrosinase had a higher affinity for DL-DOPA than for L-tyrosine. Boughilloux et al. (1963) isolated 4 different forms of tyrosinase from mushroom, all of which were capable of oxidizing DOPA more actively than tyrosine. A similar observation was also reported by Harrison et al. (1967) using a fluorescence spectrophotometric technique.

Apparent K_m values for the oxidation of L-tyrosine and DL-DOPA by mushroom tyrosinase in the presence of kojic acid were determined to be 2.02 and 0.66 mM, respectively. The inhibitor constant (K_i) was determined to be 0.03 mM for the former (competitive inhibition) and 0.02 mM for the latter (mixed-type inhibition). Since kojic acid was a competitive inhibitor, it would compete with L-tyrosine for the active site (Segel,1976). The results also showed that the inhibitory properties of

kojic acid on mushroom tyrosinase depends upon whether *o*-diphenol (DL-DOPA) or monophenol (L-tyrosine) is used as a substrate. In this study, a mixed-type inhibition implies that kojic acid affected the affinity of the enzyme for DL-DOPA but did not bind at the active site (Webb, 1963).

The K_m value for the oxidation of chlorogenic acid by potato PPO was 5.20 mM, while it was 7.89 mM for the oxidation of catechol. These values were lower than those reported by Abukharma and Woolhouse (1966), Alberghina (1964), and Macrae and Duggleby (1968). Variations in enzyme preparations and assay methods could have contributed to these differences in K_m values (Macrae and Duggleby, 1968).

In the presence of kojic acid, the apparent Michaelis constant for the oxidation of chlorogenic acid became 7.23 mM while it was 10.5 mM for catechol. The inhibitor constant for the oxidation of chlorogenic acid was 0.60 mM and 0.71 mM for catechol oxidation. Thus, kojic acid was more competitive with chlorogenic acid than with catechol for the active site. The K_i values for kojic acid using chlorogenic acid and catechol as substrate were lower than those reported for *p*-nitrophenol, ferulic acid, *p*-coumaric acid, 2,3-dihydroxy-naphthalene, and cinnamic acid (Macrae and Duggleby, 1968).

The Michaelis constants for the oxidation of 4-methylcatechol and chlorogenic acid by apple PPO were determined to be 3.85 and 8.20 mM, respectively. The K_m value for 4-methylcatechol was close to that reported by Harel et al. (1965) and Mayer et al. (1964), whereas the K_m for chlorogenic acid was higher (Walker, 1964).

When kojic acid was added, the apparent Michaelis constants for 4-methylcatechol and chlorogenic acid were changed to 4.28 and 10.3 mM, respectively. The inhibitor constant of kojic acid for 4-methylcatechol and chlorogenic acid oxidation was 0.06 and 0.13 mM, respectively. The former was similar to the K_i of 2,3-naphthalenediol (Mayer et al., 1964). However, both K_i values were lower than that of cinnamic acid (1.4 and 0.14 mM, respectively) when it was used as an inhibitor in a solubilized PPO system (Walker and Wilson, 1975). Using 4-methylcatechol, chlorogenic acid, and catechin as substrates, Walker and Wilson (1975) observed that cinnamic acid, ferulic acid, and coumaric acid derivatives behaved as competitive inhibitors of apple PPO. The Michaelis constants for the oxidation of L-DOPA and catechol by white shrimp PPO were determined to be 3.48 and 4.27 mM, respectively. The former (L-DOPA) K_m value was slightly higher than that reported for DL-DOPA (2.8 mM) by Simpson et al. (1988a). Regarding lobster PPO, the K_m values for oxidation of DL-DOPA and catechol were 3.27 and 4.98 mM, respectively. These values were lower than those reported by Chen et al. (1991a). Lobster PPO used in this study was further purified by 7.5% acrylamide gel and thus possessed a higher specific activity. For grass prawn PPO, the K_m values for DL-DOPA and catechol were 3.64 and 5.29 mM, respectively. The former value was close to that reported by Rolle et al. (1991). In comparison to pink shrimp (Simpson et al., 1988a), white shrimp, grass prawn and lobster PPO showed comparatively higher K_m values when either L-DOPA or DL-DOPA was used as substrate.

When kojic acid was added as an inhibitor, a mixed-type inhibition was observed for the oxidation of both substrates by these three

crustacean PPO. The inhibitor constant for the oxidation of DL-DOPA by grass prawn PPO was 0.05 mM and 0.07 mM for catechol. Similarly, the K_i values were determined to be 0.07 mM for DL-DOPA and 0.10 mM for catechol when lobster PPO was used. For white shrimp PPO, the K_i of L-DOPA and catechol was determined to be 0.15 and 0.18 mM, respectively. Antony and Nair (1975) studied the inhibitory effect of several chemicals on prawn phenolase and found L-tyrosine (K_i = 0.38 mM) was a competitive inhibitor to the oxidation of DOPA. In contrast, L-cysteine and sodium diethyl dithiocarbamate were found to behave as mixed-type inhibitors. Madero (1982) reported that bisulfite was a competitive inhibitor to brown shrimp PPO. The observation of lower K_i values for grass prawn and lobster PPO than white shrimp PPO further demonstrate that kojic acid exhibited a greater inhibitory effect on those two enzymes than the latter one (Figures 26, 27, and 28). Also, when the same amount of kojic acid was applied to the assay mixtures, greater inhibition was observed for DL-DOPA than for catechol (Figures 27 and 28).

It was noted that the K_m value of white shrimp PPO for DL-DOPA (3.20 mM) was close to that for L-DOPA (3.48 mM). For potato and apple PPO, both enzymes showed an extremely high affinity for DL-DOPA than for chlorogenic acid, 4-methylcatechol, and catechol (Table 4). Also, these two enzymes showed a higher affinity for DL-DOPA than other PPOs. Kojic acid also exhibited the same inhibitory mechanism on the oxidation of DL-DOPA as well as on other diphenolic substrates by white shrimp, potato, and apple PPO (Table 4). Kojic acid exerts more effective inhibition (Table 4; K_i) on DL-DOPA oxidation by potato and apple PPO than on other diphenolic substrates.

The results thus indicated that kojic acid could inhibit the oxidation of phenolic substrates by mushroom, potato, apple, white shrimp, grass prawn, and lobster PPO. Significant inhibitory effects with different types of inhibition mechanisms were observed with these PPO activities when DOPA was used as substrate. DOPA is a dominant phenolic substrate responsible for the formation of melanin (blackening spot) in crustaceans. It seems likely that kojic acid could be potentially used as an inhibitor in the prevention of melanosis in seafood products.

Effect of Pre-incubation Temperature on PPO Inhibition by Kojic Acid

The pre-incubation temperatures did not affect the inhibitory activity of kojic acid on various PPOs (Table 5). More than 75% inhibition was observed with mushroom tyrosinase and lobster PPO when 0.28 mM kojic acid was introduced into the assay mixture containing DL-DOPA and enzyme. However, only the respective 26% and 41-46% of the potato and apple PPO activity were inhibited when 0.56 mM kojic acid was added. These observations were in accordance with earlier findings that kojic acid was less inhibitory on potato and apple PPO than on mushroom and lobster PPO. Although the pre-incubation at 0°C usually gave a higher percentage of inhibition than at higher temperatures, the difference was insignificant ($P > 0.05$). Thus, kojic acid can be used to inhibit PPO at either refrigeration or ambient temperature.

Effect of Kojic Acid on the Hydroxylation Capability of PPO

The lag period associated with the hydroxylation of monohydroxyphenol by PPO increased with increasing concentrations (35.2 -

Table 5. Effect of Different Pre-incubation Temperatures on the Inhibition of Various Polyphenol Oxidases (PPO) by Kojic Acid

Enzyme source	% Inhibition		
	0°C	25°C	37°C
Mushroom	78.5	77.3	75.1
Potato	26.2	25.5	25.4
Apple	45.6	41.8	41.1
Lobster	78.1	77.0	76.8

563 μM) of kojic acid (Figure 29). Kojic acid at higher concentrations was shown to exert a profound inhibitory effect on the oxidation of L-DOPA by PPO. Thus, kojic acid did not behave as ascorbate, hydroquinone, H_2O_2, and NH_2OH that were reported to be capable of reducing the lag period of hydroxylation of monohydroxyphenol when added at small concentrations (Kahn, 1983; Kahn and Andrawis, 1986; Sato, 1969; Vaughan and Butt, 1970).

Effect of Kojic Acid on O_2 Uptake by PPO Reaction

Consumption of oxygen did not take place with kojic acid, the substrates (4-methylcatechol and chlorogenic acid for apple PPO, and DL-DOPA and catechol for lobster PPO), or the kojic acid-substrate mixtures. When PPO was added to the mixture containing substrate and buffer, O_2 consumption occurred immediately. Although O_2 uptake by the PPO-substrate mixture still took place when kojic acid was added, the percentage of O_2 consumption in these mixtures decreased with increasing concentrations of kojic acid (Table 6). For example, O_2 consumption for the oxidation of DL-DOPA by lobster PPO in the presence of 0.56 and 1.06 mM kojic acid was inhibited by 60.3 and 80.3%, respectively.

Effect of Kojic Acid on Reduction of Cu^{2+}

Bathocuproine disulfonic acid reacts with Cu^{+} to form a red color complex having an optimal absorption at 483 nm (Blair and Diehl, 1961). Thus the reducing ability of kojic acid can be determined from the measurement of the absorbance at 483 nm in a model reaction mixture. The absorbance of the reaction mixture increases with increasing concentrations of kojic acid and then reaches a plateau when kojic acid

Figure 29. Effect of Kojic Acid (●, 0.35; △, 1.41; ▲, 2.81; and □, 5.63 mM) on the Hydroxylation of Monohydroxyphenol by Mushroom Tyrosinase (PPO); Control (O) Was Run Similarly Except That Kojic Was Replaced by an Equivalent Volume of Phosphate Buffer.

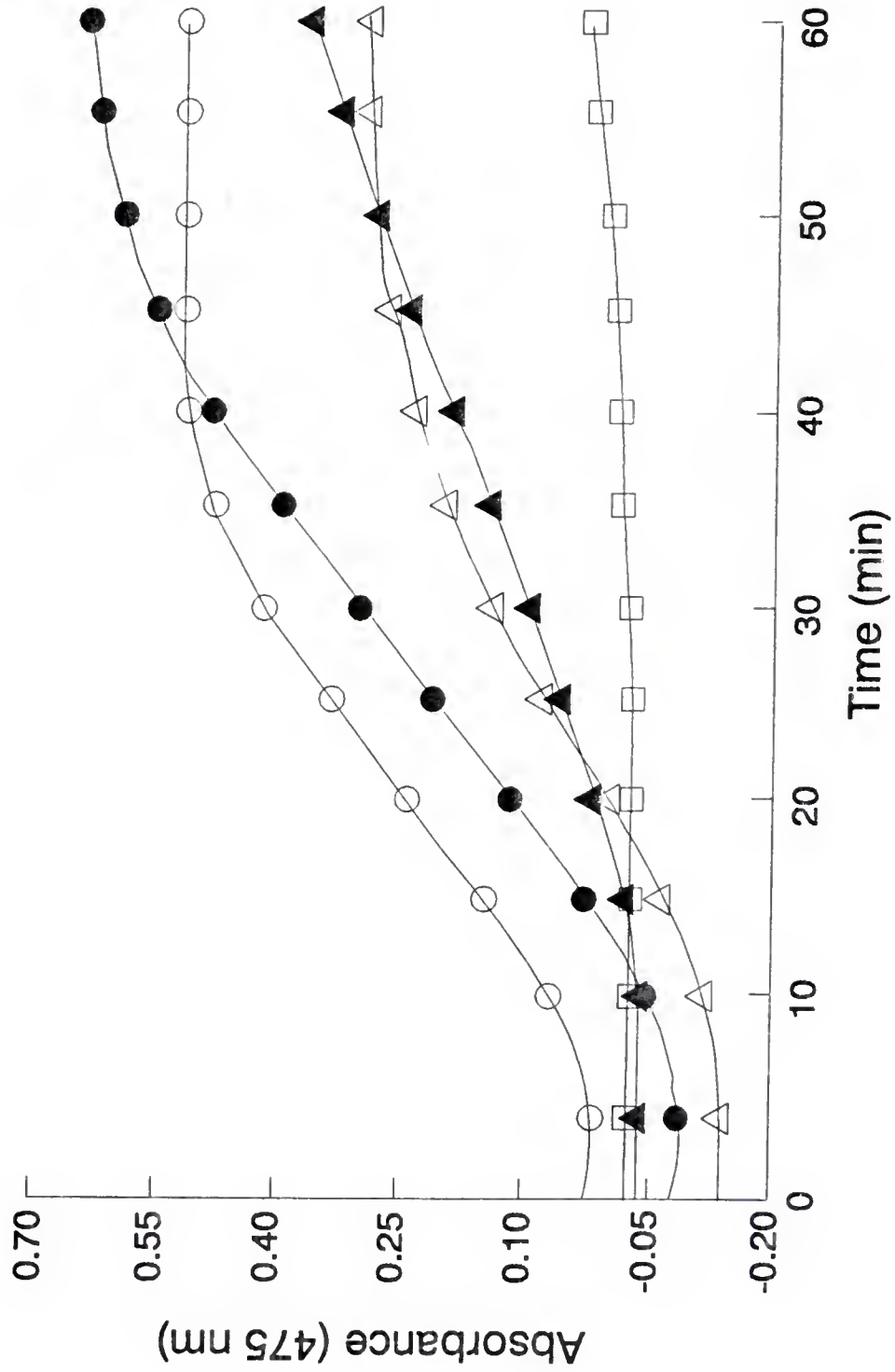

Absorbance (475 nm)
0.70
0.55
0.40
0.25
0.10
-0.05
-0.20
0
10
20
30
40
50
60
Time (min)

Table 6. Inhibitory Effect of Kojic Acid on the Consumption of Oxygen by Polyphenol Oxidase

	% Inhibition			
	Lobster PPO		Apple PPO	
Kojic acid (mM)	DL-DOPA	Catechol	4-Methylcatechol	Chlorogenic acid
0	0	0	0	0
0.28	50.2	47.4	ND	ND
0.56	60.3	65.0	36.7	24.8
1.06	80.3	77.5	50.9	41.9
1.41	ND	ND	55.4	52.1

ND: Not determined

exceeds 0.28 mM (Figure 30). On the basis of molar extinction coefficient for Cu^{+}-bathocuprione disulfonate complex (Blair and Diehl, 1961), all the Cu^{2+} present in the reaction mixture was reduced to Cu^{+} when kojic acid was added at a concentration greater than 0.28 mM.

Effect of Kojic Acid on Quinone Products

Spectrophotometric scanning of the product generated from the reaction of DL-DOPA and mushroom PPO revealed two distinct absorption peaks at 316 and 480 nm (Figure 31a). The addition of 5.63 mM kojic acid to this solution caused the color to change from red-brown to violet, and the subsequent disappearance of the 480 nm peak which represents dopaquinone (Figure 31b) (Fling et al., 1963). Similar phenomena occurred when kojic acid (5.63 mM) was added to the reaction mixture containing lobster PPO and DL-DOPA. Thus, the formation of dopaquinone from DL-DOPA through the action of PPO was affected by the presence of kojic acid.

This finding was further verified by the TLC analysis of the DL-DOPA standard and the reaction mixture (dopaquinone) containing 3.5 mM of kojic acid. A reddish-purple spot with a R_f value of 0.76 was detected for the DL-DOPA of these two samples on TLC plate following spraying with ninhydrin reagent. However, no such reddish-purple spot was detected for control sample that contained only dopaquinone.

The kojic acid effect on the quinone products formed by the action of PPO on DL-DOPA is attributed to the reduction of dopaquinones back to diphenols (DL-DOPA). Many reagents including cysteine, bisulfite, and ascorbic acid are known to retard enzymatic browning through this

Figure 30. Effect of Kojic Acid on Reduction of Cu^{2+} to Cu^{+} in a Model System; One-mL Kojic Acid (0.04 - 2.81 mM) Was Incubated with 0.5 mL of 0.4 mM $CuSO_4$ at Ambient Temperature Followed by the Addition of 0.5 mL of 4 mM Bathocuproinedisulfonic Acid. The Absorbance of the Mixture Was Measured at 483 nm.

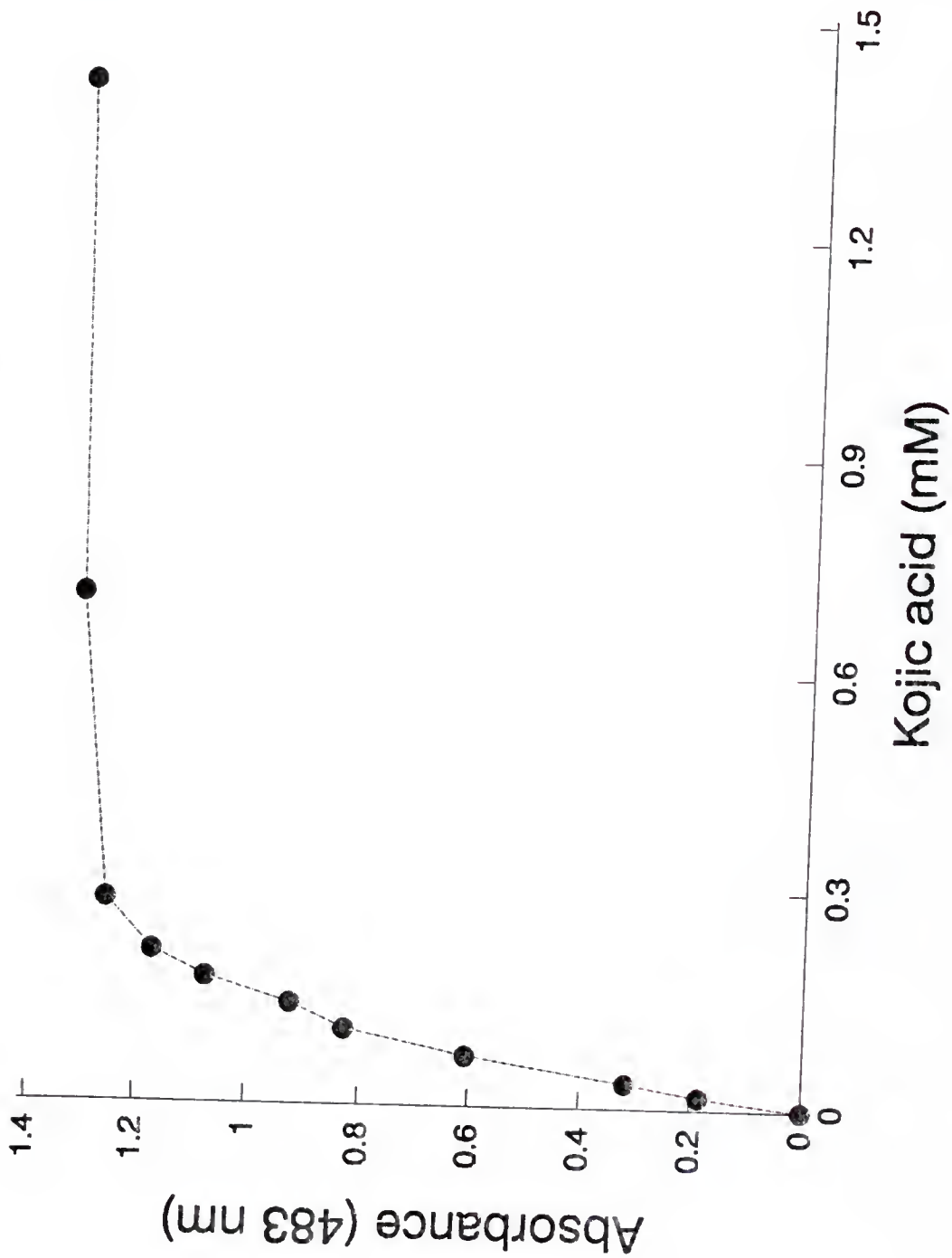
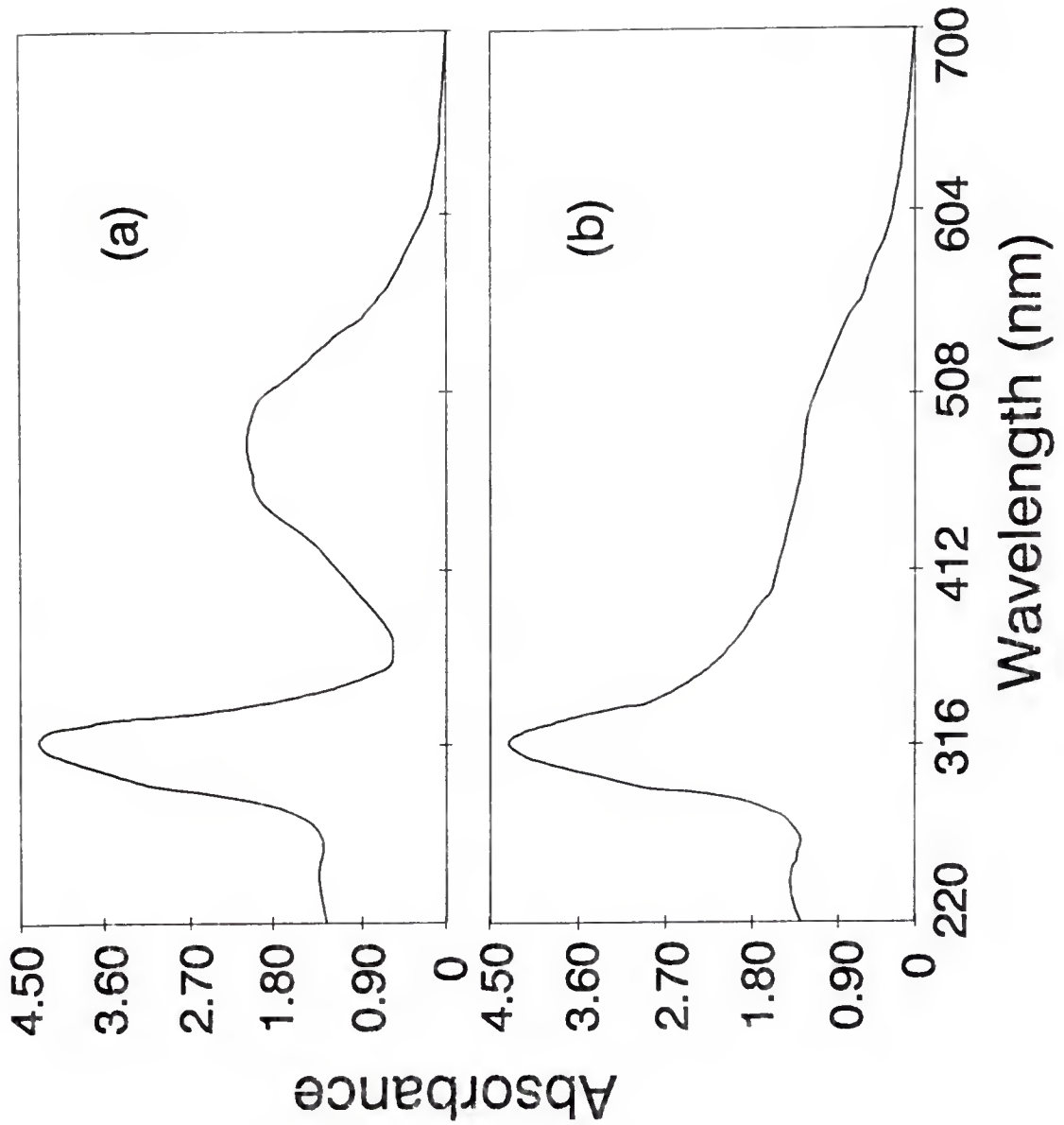

Absorbance (483 nm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
0.3
0.6
0.9
1.2
1.5
Kojic acid (mM)

Figure 31. Change in Absorption Spectra of Dopaquinone due to the Addition of 5.63 mM Kojic Acid, (a) Dopaquinone Produced from the Action of Mushroom PPO on DL-DOPA; and (b) Dopaquinone Plus Kojic Acid


(a)
(b)
4.50
3.60
2.70
1.80
0.90
0
Absorbance
220
316
412
508
604
700
Wavelength (nm)

Enzymatic Activities of Kojic Acid-treated PPO

The dialysate of the control sample and kojic acid-treated PPO preparations showed an equivalent volume. Data listed in Table 7 show that there was no significant difference ($P > 0.05$) in the enzyme activity between the control and the kojic acid-treated PPO following dialysis against phosphate buffer. The restoration of enzymatic activity was attributed to the removal of kojic acid from the enzyme mixture; the failure to detect any kojic acid residue following dialysis verified this assumption. These results thus indirectly suggested that kojic acid did not bind irreversibly toenzyme and the inhibition on PPO was reversible. Such inhibitory characteristics are in contrast to that of sulfite and its derivatives (Sayavedra-soto and Montgomery, 1986; Ferrer et al., 1989b).

Conclusion

Kojic acid exhibited a competitive inhibition for the oxidation of chlorogenic acid and catechol by potato PPO and of 4-methylcatechol and chlorogenic acid by apple PPO. This compound showed a mixed-type inhibition for white shrimp, grass prawn, and lobster PPO when DL-DOPA and catechol were used as substrates but a mixed-type and a competitive inhibition for mushroom PPO when DL-DOPA and L-tyrosine were used, respectively. This compound also showed a competitive inhibition on the oxidation of DL-DOPA by apple and potato PPOs. The competitive inhibition suggests that kojic acid might be a nonmetabolizable analog of the substrates (DL-DOPA, chlorogenic acid, or 4-methylcatechol). The result also implies that apple, potato, and/or mushroom PPO bear the structural

Table 7. Enzymatic Activity of Kojic Acid-treated Polyphenol Oxidase Following Kojic Acid Removal[a]

Enzyme	Control	Kojic acid (mM)		
		0.56	1.12	5.60
Mushroom[b]	0.80[d]	0.80	0.79	ND[e]
Lobster[b]	0.124	0.118	0.120	ND
White shrimp[b]	0.032	0.031	0.032	ND
Potato[c]	0.059	ND	0.060	0.061
Apple[c]	0.183	ND	0.188	0.187

[a]Enzyme was incubated with kojic acid for 30 min followed by dialyzing against phosphate buffer.

[b]DL-DOPA was used as substrate and the activity was determined as ΔA_{475} nm/min.

[c]Catechol was used as substrate and the activity was determined as ΔA_{395} nm/min.

[d]Result was an average of three observations; values within the same row are not significantly different ($P > 0.05$) from each other.

[e]ND, not determined

similarities on their active site. The inhibitory mode of kojic acid on PPO was shown following actions: (1) by competing with substrate for the active site of the enzyme; (2) by interfering with the uptake of O_2 required for the enzymatic reactions; (3) by reducing *o*-quinones to *o*-diphenols to prevent melanin formation via polymerization; and/or (4) by combination of the above three methods.

EFFECT OF CARBON DIOXIDE ON THE INACTIVATION OF PLANT AND CRUSTACEAN POLYPHENOL OXIDASES

Introduction

Undesirable enzymatic browning caused by polyphenol oxidase (E.C. 1.14.18.1; PPO) on the surface of food products has been of great concern to food scientists and food processors since the melanin formed reduces the consumers' acceptability of these products. Many chemicals have been studied extensively for their effectiveness in inhibiting PPO activity (Jones et al., 1965; Mayer et al., 1964; Palmer and Roberts, 1967; Walker, 1975; Madero and Finne, 1982; Golan-Goldhhirsh and Whitaker, 1984; Sayavedra-Soto and Montgomery, 1986; Ferrer et al., 1989b; Chen et al., 1991b) and shown to effectively inhibit melanosis of fruits, vegetables, and crustaceans. However, problems related to off-flavor, off-odor, toxicity, and economic feasibility affect the application of these compounds (Eskin et al., 1971).

A modified atmosphere with carbon dioxide has been used as a physical application to affect many enzyme activities (Parkin and Brown, 1982; Gee and Brown, 1978a, b), including the inhibition of PPO and thus the prevention of discoloration in fruits and vegetables (Kader et al., 1973). Supercritical (SC) fluids exhibiting physicochemical properties intermediate between those of liquids and gases has been reported by several workers for its inactivation of peroxidase (Christianson et al., 1984), PPO (Zemel, 1989), and pectinesterase (Arreola, 1990) when carbon

dioxide (CO_2) was used as the fluid. CO_2 is used in SC fluid because it is nontoxic, nonflammable, inexpensive, and readily available (Hardardottir and Kinsella, 1988). CO_2 has a relatively low critical temperature and pressure (Rizvi et al., 1986). Taniguchi et al. (1987) studied the retention of alpha-amylase, glucose oxidase, lipase, and catalase activity by SC-CO_2. Although SC-CO_2 has been shown to inactivate PPO, information regarding the inhibitory effect and the inactivation kinetics of SC-CO_2 on purified PPO has not been elucidated. This study was undertaken to investigate the effect of CO_2 (1 or 58 atm) on the inactivation of Florida spiny lobster, brown shrimp, and potato PPO.

Materials and Methods

Fresh Florida spiny lobster (*Panulirus argus*) tails were obtained from the Whitney Marine Laboratory and transported in ice to the laboratory and stored at -20°C. Russet potato tuber was purchased from a local supermarket. Non-sulfited fresh brown shrimp (*Penaeus aztecus*) were obtained from a local seafood store. Lobster cuticle, shrimp cephalothorax (head), and potato peel were frozen in liquid nitrogen and ground into a fine powder using a Waring blender. The ground powder was stored at -20°C until needed.

Extraction and Purification of Lobster, Shrimp, and Potato PPO

PPO was extracted and purified following the procedures of Chen et al. (1991a). Ground powder was added to 0.05 M sodium phosphate buffer (pH 7.2) (1:3, w/v) containing 1 M NaCl and 0.2% Brij 35, stirred for 0.5 hr at 4°C, and then centrifuged at 8,000*g* (4°C) for 30 min. The

supernatant was dialyzed at 4°C overnight against 3 changes of 4L distilled water.

Crude PPO preparation was purified further using a nondenaturing preparative polyacrylamide gel electrophoresis (PAGE) system. Equipment utilized included a gel tube chamber (Model 175, Bio-Rad) and a power supply (Model EPS 500/400, Pharmacia). A one-mL aliquot of crude enzyme extract (lobster, shrimp, or potato) was applied to each of eight gel tubes (1.4 cm I.D. x 12 cm length) containing 5% acrylamide/ 0.13% bisacrylamide gel and run at a constant current of 10 mA/tube (Sigma Chemical CO., 1984). PPO was visualized using a specific enzyme-substrate staining method; 10 mM DL-β-3,4-dihydroxyphenylalanine (DL-DOPA) in 0.05 M sodium phosphate buffer (pH 6.5) was used as substrate. After the migration of the enzyme relative to the dye front (R_f) was determined using one of the eight gels, the remaining gels were sectioned at the determined R_f. PPO was eluted from the gel by homogenization in distilled water utilizing a Dounce manual tissue grinder. The homogenates were filtered through Whatman No. 4 filter paper, pooled, and concentrated using an Amicon stirred cell (Model 8050) fitted with a YM 10 filter. Concentrated PPO was dialyzed overnight (4°C) against 2 changes of 4L distilled water.

Protein Quantitation and Enzyme Purity Determination

Protein contents of various PPO preparations were quantitated using the Bio-Rad Protein Assay kit with bovine serum albumin as standard. Enzyme purity was examined using a mini gel system (Mini-Protean II Dual Slab Cell) (Bio-Rad, 1985b). PPO (20 μg protein/well) was loaded and electrophoresis was carried out at constant voltage (200 V) for 35 min.

Purity of the preparations was determined by comparing gels stained with 10 mM DL-DOPA in 0.05 M sodium phosphate buffer (pH 6.5) and then with a Coomassie blue R-250 solution.

Enzyme Activity assay

PPO activities were measured by adding 60 μL of the preparation to 840 μL 10 mM DL-DOPA in 0.05 M sodium phosphate buffer (pH 6.5) and monitored at 25°C for 5 min. Maximal initial velocity was determined as $\Delta A_{475\ nm}$/min and one unit of PPO activity was defined as an increase in absorbance of 0.001/min at 25°C. Unless otherwise specified, experiments were replicated three times. The enzyme activities of lobster, brown shrimp, and potato PPO was determined as 3,750, 740, and 5,400 units/mg protein, respectively.

Effect of CO_2 (1 atm) on PPO Activity

Three batches of 20-mL lobster PPO (1,470 units/mg protein) were loaded in 50-mL polymethylpentene tubes (Nalgene Co.) and were heated respectively at 33°, 38°, and 43°C in a water-bath. Following the equilibration of the PPO solution to the desired temperatures, liquid carbon dioxide [Coleman grade (99.99% CO_2), Liquid Air Co., Walnut, CA)] at a flow rate of 110 mL/min was bubbled through the PPO solution for 30 min. PPO (500 μL) was removed every 5 min from the stock solution heated at 33° or 38°C. PPO (500 μL) heated at 43°C was sampled every min during the first 5 min and then 5 min thereafter. PPO samples were put in 1.5 mL microcentrifuge tubes (Fisher) and immediately cooled by emerging the tube in an ice bath. Following equilibration to ambient temperature, PPO

activity was determined by adding 60 μL PPO into a microcuvette containing 840 μL 10 mM DL-DOPA in 0.05 M sodium phosphate buffer (pH 6.5). The reaction was monitored at 475 nm (25°C) for 5 min. The activity of N_2-treated and non-gas-treated controls heated at 33°, 38°, or 43°C were also determined as previously described. Percentage of relative activity was determined as $(E_t/E_o) \times 100$, where E_t and E_o were the PPO activities at time t and original activity, respectively. Changes in pH resulting from CO_2 treatment was monitored using a digital pH meter (E632, Metrohm Ltd., Switzerland) equipped with a microelectrode.

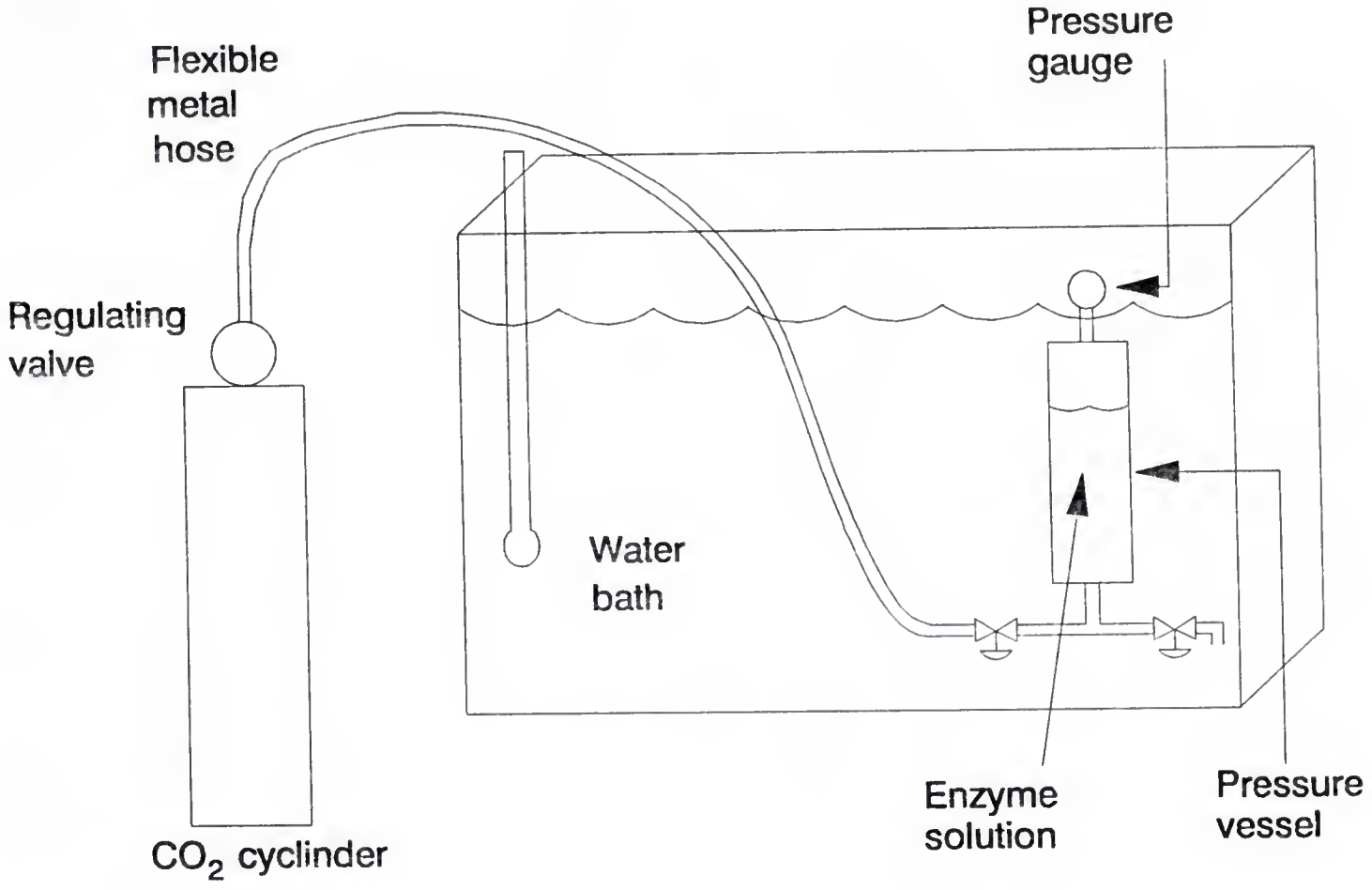
pH Control Study

Mixtures containing 1 mL PPO preparation and 3 mL $NaHCO_3$ (pH 5.3) were heated for 30 min at 33°, 38°, and 43°C, respectively, in a water bath. The mixture was then instantly removed and emerged into a 0°C ice chest. Following equilibration to ambient temperature, PPO activity was assayed as previously described.

Effect of high pressure CO_2 on PPO activity

The apparatus used for PPO inactivation by high pressure CO_2 is shown in Figure 32. CO_2 was connected to a high-pressure resistant stainless steel vessel (volume = 100 mL) equipped with valves through a metal hose. After the vessel was immersed into a water bath maintained at 43°C, a constant pressure of 850 psi (58 atm) inside the vessel chamber was achieved by adjusting the pressure-regulating valve. For each study, 80 mL lobster, brown shrimp, or potato PPO was placed in the vessel. After

Figure 32. Apparatus Used for Studying Polyphenol Oxidase (PPO) Inactivation by High Pressure CO_2


Flexible
metal
hose
Pressure
gauge
Regulating
valve
Water
bath
Enzyme
solution
Pressure
vessel
CO_2 cyclinder

treatment parameters were equilibrated to the desired conditions, PPO (8 mL) was sampled after the vessel was removed from the water bath. Each time after sampling, the vessel was replaced into the bath. Sampling was done every minute during the first 5 min and then every 5 min thereafter.

Following equilibration to ambient temperature, PPO activity was determined as previously described. The activity for temperature control treatment was also determined. Percentage of relative activity was determined as $(E_t/E_o) \times 100$, where E_t and E_o were the PPO activities at time t and the original activity without heat and CO_2, respectively. The pH change resulting from CO_2 treatment during the course of this study was monitored using a pH meter.

Kinetics of PPO Inactivation

The inactivation reaction constant (k) and the activation energy (E_a) of PPO in the presence or absence of CO_2 (1 atm) were determined according to the Arrhenius equation by measuring the initial rate at different temperatures and plotting the logarithmic value of V_{max} versus 1/T (Segel, 1976). The D value (decimal reduction value) defined as the time required to inactivate 90% of the original enzyme activity at a constant temperature was determined from the negative reciprocal of the slope from a plot of logarithmic value of enzyme activity versus time (Richardson and Hyslop, 1985). The z value which is the number degrees required for the thermal inactivation curve to traverse one logarithmic cycle was determined by plotting the logarithmic value of (D_2/D_1) versus (T_2-T_1), where D_2 and D_1 were the D values at temperature T_2 and T_1 (fahrenheit, ^oF), respectively (Richardson and Hyslop, 1985).

Nondenaturing Polyacrylamide Gel Electrophoresis of CO_2 (1 atm)-treated PPO

Mini polyacrylamide gel with a dimension of 7 x 8 cm (1 mm thickness) and containing 7.5% acrylamide/ 0.2% bisacrylamide was prepared according to the Mini-Protean II Dual Slab Cell Instruction Manual (Bio-Rad, 1985b). After 50 μL PPO was loaded onto the sample well, the electrophoresis was carried out at a constant voltage of 200 V for 35 min. Following electrophoresis, the gel was stained with 0.1% Coomassie blue R-250 in a fixative solution (40% MeOH and 10% HOAc, v/v) for 0.5 hr and then destained. The molecular weights of PPO were determined by comparing the R_f values of protein with those of nondenaturing protein molecular weight standards (Sigma) containing α-lactalbumin (14 kD), bovine erythrocytes carbonic anhydrase (29 kD), chicken egg albumin (45 kD), bovine serum albumin (monomer, 66 kD; dimer, 132 kD), and jack bean urease (dimer, 240 kD; tetramer, 480 kD).

Mass Balance of High Pressure CO_2-treated and Nontreated PPO

The high pressure CO_2-treated PPO solution (1.5 mL) was centrifuged in an Eppendorf 5415 microcentrifuge (Brinkmann Instruments Inc., Hamburg, Germany) at 13,000 rpm for 30 min. After the supernatant was collected, the pellet was redissolved in 0.5 mL 0.05 M sodium phosphate buffer (pH 6.5), and the protein contents of both portions quantitated using the Bio-Rad Protein Assay kit. The combined protein contents of both portions were then compared to that of an equal volume (1.5 mL) of nontreated PPO.

The protein patterns of high pressure CO_2-treated and untreated PPO were also analyzed using mini sodium dodecyl sulfate (SDS) polyacrylamide gel. Fifty-μL supernatant and pellet portions of high pressure CO_2-treated

and nontreated PPO were loaded individually onto the sample wells. Electrophoresis was carried out at a constant voltage of 200 V for 35 min. Following electrophoresis, the gel was stained with the silver stain kit (Bio-Rad). The SDS-6H standard (Sigma) containing carbonic anhydrase (29 kD), egg albumin (45 kD), bovine albumin (66 kD), phosphorylase B (97.4 kD), β-galactosidase (116 kD), and myosin (205 kD) was run together with the samples for protein molecular weight determination.

Polyacrylamide Gel Isoelectric Focusing of CO_2-treated PPO

A gel mixture containing 4% acrylamide, 2.5% Triton X-100, 8 M urea, and 5.5% ampholyte (Pharmalyte 3-10, Pharmacia) was degassed for 5 min. After the addition of 5% (v/v) fresh ammonium persulfate and 0.1% (v/v) N,N,N′,N′-tetramethylethylenediamine (TEMED), the gel mixture was poured into a 16 x 20 cm slab gel plates assembled with a 0.75 mm comb and allowed to polymerize for 1.5 hr according to the Protean II Slab Cell Instruction Manual (Bio-Rad Labs., 1985a). Following the removal of the comb, buffer containing 0.2% (v/v) Pharmalyte 3-10 and 5% (v/v) Triton X-100 was overlayed onto the polymerized gels and allowed to sit for 1 hr. Prefocusings at constant voltages of 200 V (15 min), 300 V (30 min), and 400 V (30 min) were alternately carried out after the overlaying buffer was changed (An et al., 1989). A 50 μL of CO_2 (1 or 58 atm)-treated PPO was then loaded into the sample well and electrofocusing was performed at a constant voltage of 400 V for 17 hr (An et al., 1989). The gel was fixed with the fixative solution (sulfosalicylic acid/ trichloroacetic acid/ methanol, 4:12.5:30, v/v) and then stained with Coomassie blue R-250. The isoelectric point (pI) of PPO was determined by comparing the R_f

value of the sample with those of the protein standards (Broad pI Kit, pH 3-10, Pharmacia) containing amyloglucosidase, pI 3.50; soybean trypsin inhibitor, pI 4.55; β-lactoglobulin, pI 5.20; bovine carbonic anhydrase, pI 5.85; human carbonic anhydrase B, pI 6.55; horse myoglobin-acidic band, pI 6.85; -basic band, pI 7.35; lentil lectin-acidic band, pI 8.15; -middle band, pI 8.45; -basic band, pI 8.65; and trypsinogen, pI 9.30.

Spectropolarimetric Analysis of PPO

Circular dichroic (CD) spectra of high pressure CO_2-treated and non-CO_2-treated PPO were determined at the far UV range (250 - 200 nm) using a Jasco J-20 automatic recording spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan), using a 1.0-cm Suprasil (Helma Cells) cuvette with 1.0-cm light path. Four-mL PPO (10 μg/mL) in 0.05 M sodium phosphate buffer (pH 6.5) was used as sample and the measurement of CD spectra was carried out at ambient temperature. Secondary structure calculations were performed by computer analysis of the CD spectra using the SSE program (Japan Spectroscopic Co., 1985) with myoglobin, cytochrome c, ribonuclease A, lysozyme, and papain as CD references.

Study of Restoration of CO_2-treated PPO Activity

To examine the reactivation ability of PPO following CO_2 (1 or 58 atm) treatment, a portion of CO_2-treated sample was stored at -20°C in a microcentrifuge tube (1.5 mL) for 6 weeks. After thawing at ambient temperature, the pH was then measured using a digital pH meter. Enzyme activity was determined as previously described and the assays were performed weekly. Percentage relative activity was determined as (ER_t/ER_o)

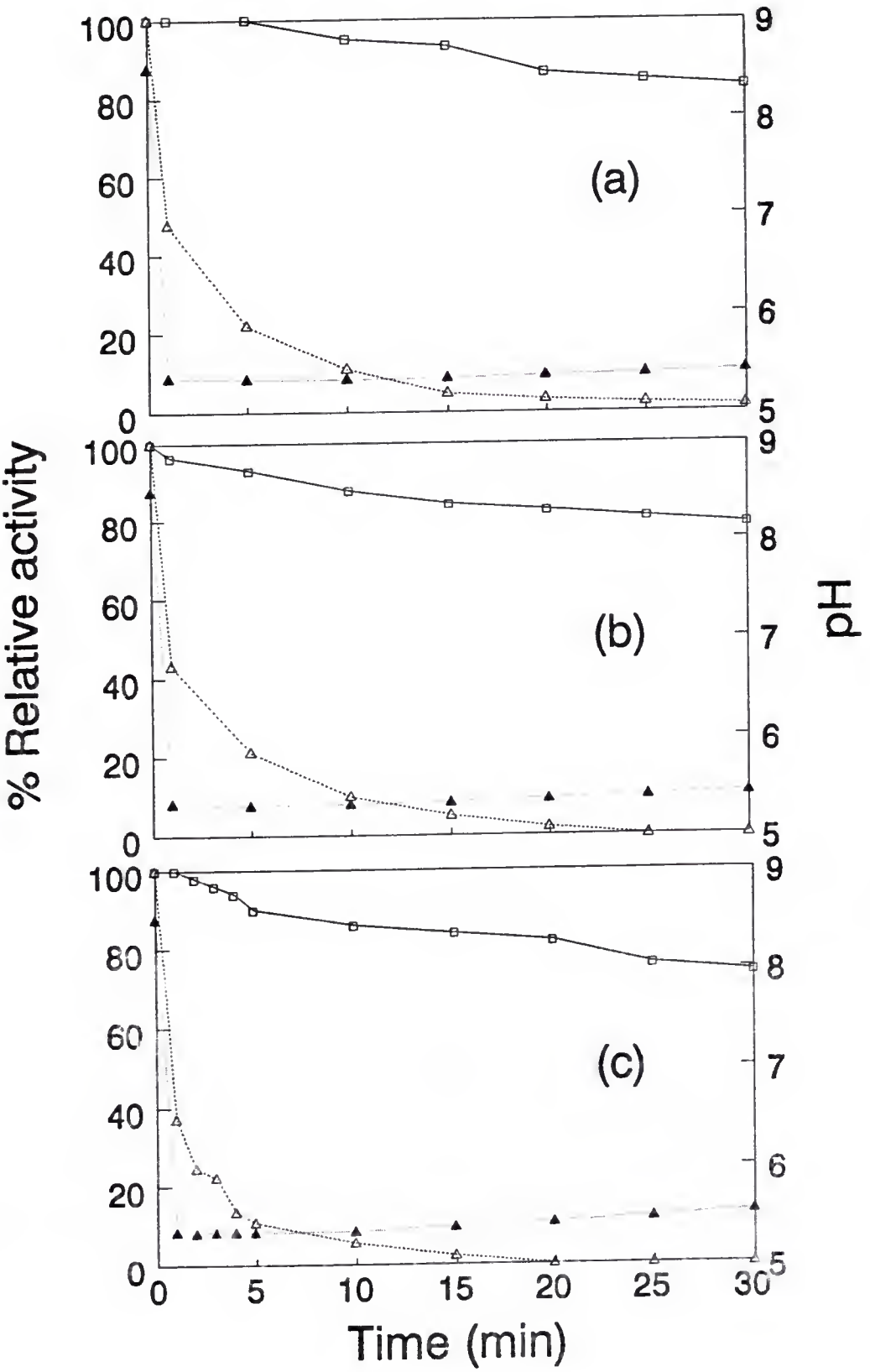
x 100, where ER_t and ER_o were the activities of CO_2 (1 or 58 atm)-treated PPO stored at time t and the original activity of non-CO_2-treated PPO, respectively.

Results and Discussion

Effect of CO_2 (1 atm) on PPO Activity

Enzyme activity of untreated PPO incubated at 33°, 38°, and 43°C decreased slightly with increased heating times (Figures 33a, 33b, and 33c). If the protein molecule absorbs too much thermal energy, the secondary and/or tertiary structure will become disrupted and enzyme will be denatured and lose its catalytic activity (Segel, 1976). PPO (43 μg protein/mL) solutions exposed to CO_2 under similar heating conditions dramatically lost their catalytic activities (Figures 33a, 33b, and 33c). Only 1.5% of the original activity remained after PPO was treated with CO_2 at 33°C for 30 min (ΔA_{475nm}/min = 0.0009 vs. 0.059) (Figure 33a). For those samples subjected to CO_2 at 38° and 43°C, no enzyme activity was detected after 25 and 20 min, respectively (Figures 33b and 33c). The spiny lobster PPO was more vulnerable to CO_2 than corn germ peroxidase (Christianson et al., 1984) and nine commercial enzyme preparations including α-amylase, glucoamylase, β-galactosidase, glucose oxidase, glucose isomerase, lipase, thermolysin, alcohol dehydrogenase, and catalase (Taniguchi et al., 1987).

Figure 33. Effect of Carbon Dioxide (1 atm) on the Change in pH (▲) and Enzyme Activity (△) of Florida Spiny Lobster PPO Incubated at 33° (a), 38° (b), or 43°C (c); the Thermal Effect on the PPO Activity in the Absence of CO_2 (□) Was Also Conducted.


% Relative activity
pH
Time (min)
(a)
(b)
(c)
100
80
60
40
20
0
9
8
7
6
5
0
5
10
15
20
25
30

Profiles of the time-related pH changes among these CO_2-treated PPO were similar (Figures 33a, 33b, and 33c). A sharp drop in pH from 8.5 to 5.3 occurred after the PPO solutions were bubbled with CO_2 for 1 min. The pH remained constant at around 5.4 for the duration of the experiment.

The pK_a value for the equilibrium between dissolved CO_2 and H^+ and HCO_3^- is 6.1. (Montgomery and Swenson, 1969). According to the Henderson-Hasselbalch equation, the molar concentration of HCO_3^- to CO_2 in solution was reduced from 251 to 0.16 when the pH of the solution dropped from 8.5 to 5.3. This study showed that exposure to CO_2 yielded a lower pH environment and resulted in a rapid acidification (Aickin and Thomas, 1975; Thomas and Ellis, 1976).

When the change in enzyme activity was compared to the change in pH among the CO_2-treated PPO solutions, it was noted that PPO activities decreased with an increase in CO_2 treatment time. The treatment of PPO solution with CO_2 caused an instant drop in pH and it then remained constant at 5.3 after 1 min. Using the pH control study, it was found that enzymes under an environmental pH of 5.3 still had 66, 60, and 35% of original activity after heating at 33°, 38°, and 43°C, respectively, for 30 min. Results from this study thus demonstrate that the loss in activity was not due entirely to pH changes.

Effect of N_2 on PPO Activity

Nitrogen gas did not inactivate PPO activity. In contrast, the relative activity of N_2-treated PPO increased with time (Table 8). Such significant ($P < 0.05$) increase in enzyme activity accompanied by a decrease in volume and thus an increase in protein concentration were

Table 8. Effect of Nitrogen on Florida Spiny Lobster Polyphenol Oxidase Activity

	% Relative activity ($\Delta A_{475\ nm}$/min)#*			
	Heating time (min)			
Temperature (°C)	5	10	20	30
33	99[a]	100[a]	110[a]	129[a]
38	107[b]	129[b]	232[b]	378[b]
43	107[b]	128[b]	176[c]	317[c]

The reaction mixture contained 0.84 mL of 10 mM DL-DOPA in 0.05 M sodium phosphate buffer (pH 6.5) and the assay was monitored at 475 nm (25°C) for 10 min; the maximal initial rate for the control of this study was 0.059.

* There are no significant differences ($P > 0.05$) among the treatments within the same column with the same superscript letter.

observed for PPO samples heated at 38° and 43°C after 20 min (Table 8). Thus, the loss in enzyme activity of CO_2-treated PPO was not due to the purging effect from the bubbling gas.

Effect of High Pressure CO_2 on PPO Activity

Heating of lobster and brown shrimp PPO at 43°C for 30 min caused some loss of enzyme activity (Figures 34 and 35). Such treatment, however, caused only 5% loss of potato PPO activity (Figure 36). No protein precipitation occurred in treated samples.

Treatment of these PPO with high pressure (58 atm) CO_2 at 43°C, however, caused a dramatic loss of enzyme activity (Figures 34, 35 and 36). Lobster, brown shrimp, and potato PPO, after treatment for 1 min, retained only 2 ($\Delta A_{475\ nm}$/min = 0.001 vs. 0.083), 22 (0.010 vs. 0.046), and 45% (0.240 vs. 0.540), of the original enzyme activity, respectively. Extended treatment of lobster and brown shrimp PPO for more than one min caused a complete loss of enzyme activity. For these two PPOs, the treatment for 10 and 15 min respectively caused protein precipitation. High pressure CO_2 treatment thus caused PPO denaturation and the loss of lobster and brown shrimp enzyme activity. The results also showed that brown shrimp PPO was slightly more resistant than lobster PPO to high pressure CO_2 treatment at 43°C, and potato PPO was the most resistant of the three. Potato PPO eventually lost 91% of its original activity after treatment for 30 min (Figure 36). Florida spiny lobster PPO was more susceptible to high pressure CO_2 than atmospheric CO_2; treatment of this PPO for 20 min with atmospheric CO_2 (1 atm) at 43°C did not cause complete loss of the enzyme activity (Figure 33c). In comparison with the studies

Figure 34. Effect of High Pressure (58 atm) Carbon Dioxide on the Change in pH (●) and Enzyme Activity (○) of Florida Spiny Lobster PPO Incubated at 43°C; (□) Represents the Activity of PPO Incubated at 43°C in the Absence of CO_2.

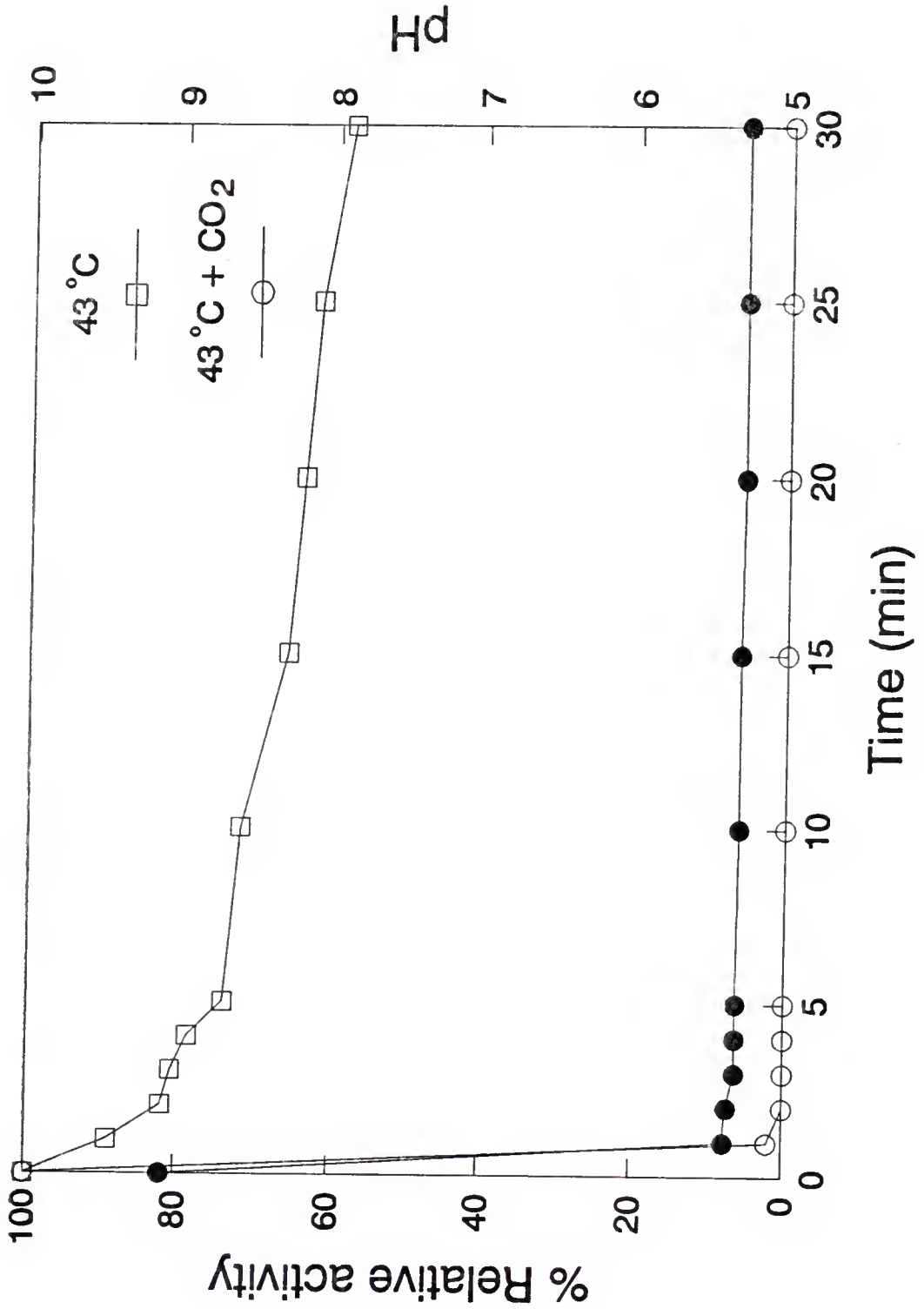

% Relative activity
100
80
60
40
20
0
Time (min)
0
5
10
15
20
25
30
pH
10
9
8
7
6
5
43 °C
43 °C + CO2

Figure 35. Effect of High Pressure (58 atm) Carbon Dioxide on the Change in pH (●) and Enzyme Activity (○) of Brown Shrimp PPO Incubated at 43°C; (□) Represents the Aactivity of PPO Incubated at 43°C in the Absence of CO_2.

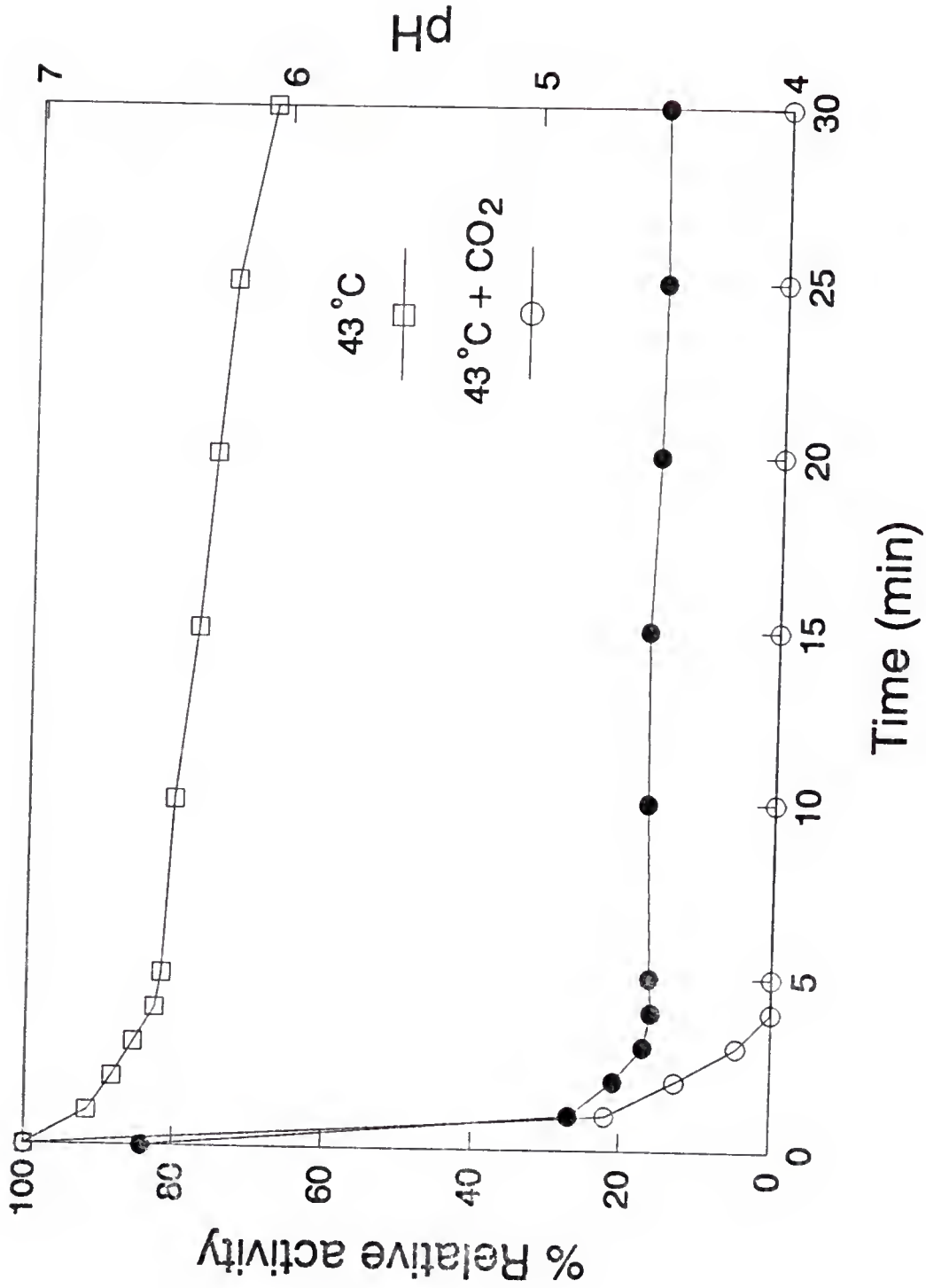
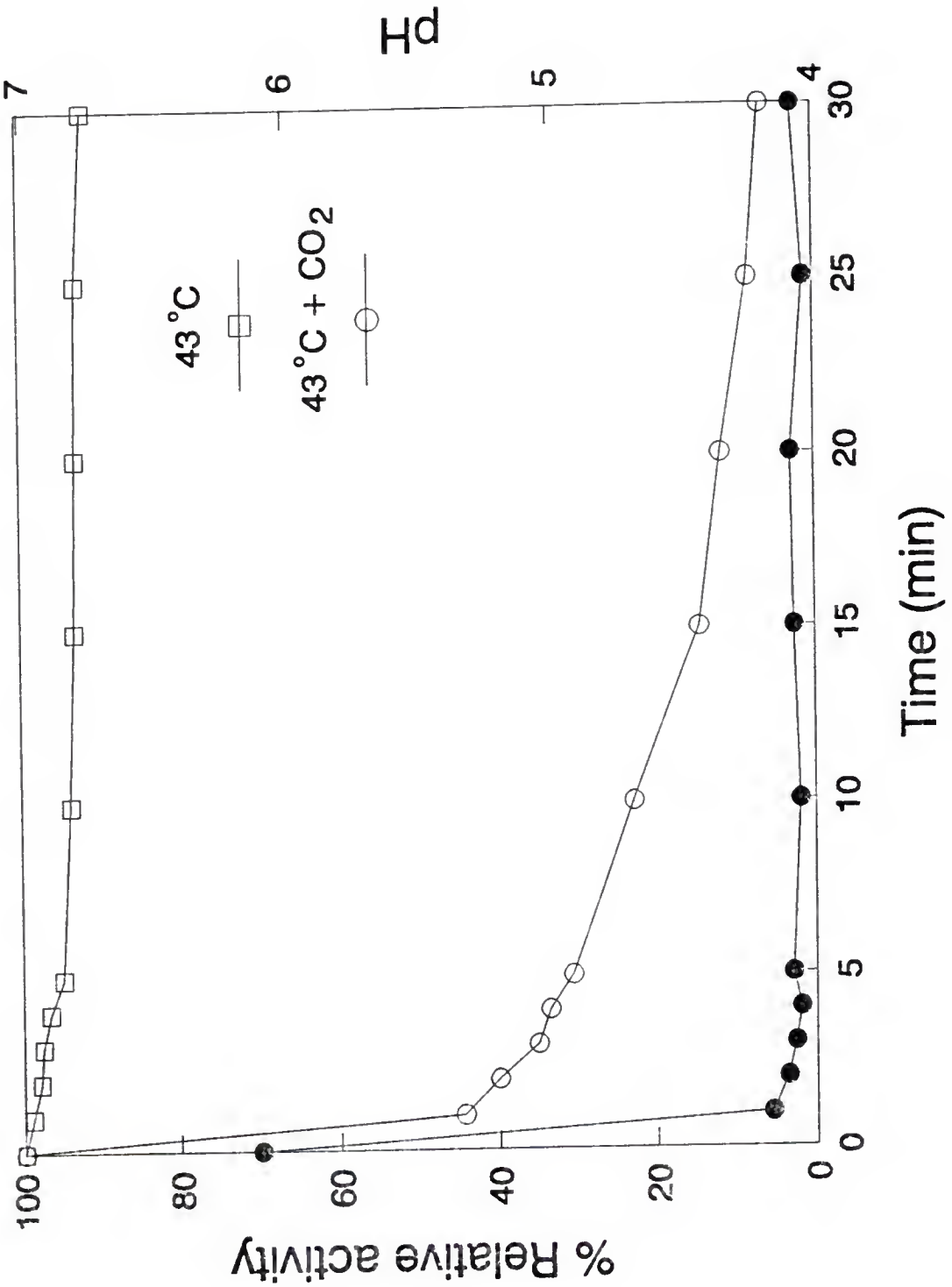

% Relative activity
100
80
60
40
20
0
pH
7
6
5
4
Time (min)
0
5
10
15
20
25
30
43 °C
43 °C + CO2

Figure 36. Effect of High Pressure (58 atm) Carbon Dioxide on the Change in pH (●) and Enzyme Activity (○) of Potato PPO Incubated at 43°C; (□) Represents the Activity of PPO Incubated at 43°C in the Absence of CO_2.


% Relative activity
100
80
60
40
20
0
0
5
10
15
20
25
30
Time (min)
pH
7
6
5
4
43 °C
43 °C + CO2

of Christianson et al. (1984) and Taniguchi et al. (1987), it also appears that lobster PPO was more vulnerable to high pressure CO_2 treatment than corn germ peroxidase, α-amylase, glucoamylase, β-galactosidase, glucose oxidase, glucose isomerase, lipase, thermolysin, alcohol dehydrogenase, and catalase.

High pressure CO_2 treatment at 43°C of the three PPO systems for 1 min caused a sharp drop in pH from 9.1 to 5.4 for lobster, 6.5 to 4.8 for brown shrimp, and 6.1 to 4.2 for potato (Figures 34, 35, and 36). The pH of the treated lobster, brown shrimp, and potato PPO systems remained constant at 5.3, 4.5, and 4.1, respectively, throughout the experiment. Overall, there was no difference in the profiles of the time-related pH change between the high pressure and atmospheric CO_2-treated PPO's (Figures 33a, 33b, and 33c).

Kinetics of PPO Inactivation

Kinetic parameters for PPO inactivation by CO_2 (1 atm) are given in Table 9. The reaction constants for lobster CO_2-treated PPO at various temperatures were comparatively higher than those for PPO with no CO_2 treatment. PPO exposed to CO_2 showed a higher activation energy (E_a = 39.7 KJ/mole) for inactivation than control (E_a = 26.6 KJ/mole) for the DOPA reaction. The D values of PPO under CO_2 treatment are lower than those of temperature controls indicating that at the temperature range used, it is much easier to inactivate PPO by CO_2 and heat than by heat alone. The smaller z values obtained for CO_2-treated samples also implies that the enzyme is more sensitive to elevated temperatures when in a CO_2 environment. The reaction constants (k) for brown shrimp and potato PPO

Table 9. Kinetic Parameters of Florida Spiny Lobster Polyphenol Oxidase Inactivation by Carbon Dioxide (1 atm) at Various Temperatures

Temperature (°C)	D (min)	Z (°C)	k (min^{-1})	E_a (KJ/mole)
Control				
33	320		7.2×10^{-3}	
38	314	69.1	7.3×10^{-3}	26.6
43	229		1.0×10^{-2}	
CO_2-treatment				
33	17.4		1.2×10^{-1}	
38	13.1	43.3	1.6×10^{-1}	39.7
43	10.2		1.9×10^{-1}	

at 43°C were determined to be 1.6×10^{-2}, 9.4×10^{-3}, and 2.5×10^{-3} min^{-1}, respectively. Under similar heating conditions in the presence of CO_2 (58 atm), the k values for brown shrimp and potato PPO were shifted to 0.98 and $6.9 \times 10^{-1} min^{-1}$. Potato PPO was more resistant to the treatments than lobster and brown shrimp PPO. This study also demonstrates that PPO was more susceptible to the combined treatment of high pressure CO_2 and heat than by heat alone.

Polyacrylamide Gel Electrophoresis of CO_2 (1 atm)-treated PPO

Only one single protein band was observed for the control and CO_2-treated PPO on the nondenaturing PAGE gel (Figure 37). No difference in protein band position on the gel occurred among the control and CO_2-treated groups. The molecular weight of this protein band was assessed as 255 kD which is close to the total molecular weights (277 kD) of three isoforms previously reported for spiny lobster PPO (Chen et al., 1991a). In addition, it is apparent that no alteration of the intact protein molecule occurred when subjected to CO_2 treatment.

Mass Balance of High Pressure CO_2-treated and Non-CO_2-treated PPO

Protein precipitates occurred after lobster and shrimp PPO were subjected to high pressure CO_2 treatment. The combined protein contents in the supernatant and pellet portions of the treated samples were close to that of their respective untreated control (Table 10). Since CO_2-treated potato PPO did not have a precipitate, no protein content was detected in the pellet portion after centrifugation (Table 10).

Figure 37. Nondenaturing Polyacrylamide Gel Electrophoresis (PAGE, 7.5% Gel) Profile of Carbon Dioxide (1 atm)-treated Florida Spiny Lobster (FSL) PPO; FSL (C) Represents Non-CO_2-treated PPO, While FSL (I), FSL (II), and FSL (III) Represent the PPO Heated at 33°, 38°, and 43°C, Respectively, in the Presence of CO_2. Numerical Designations Represent Molecular Masses of the Standard Proteins.

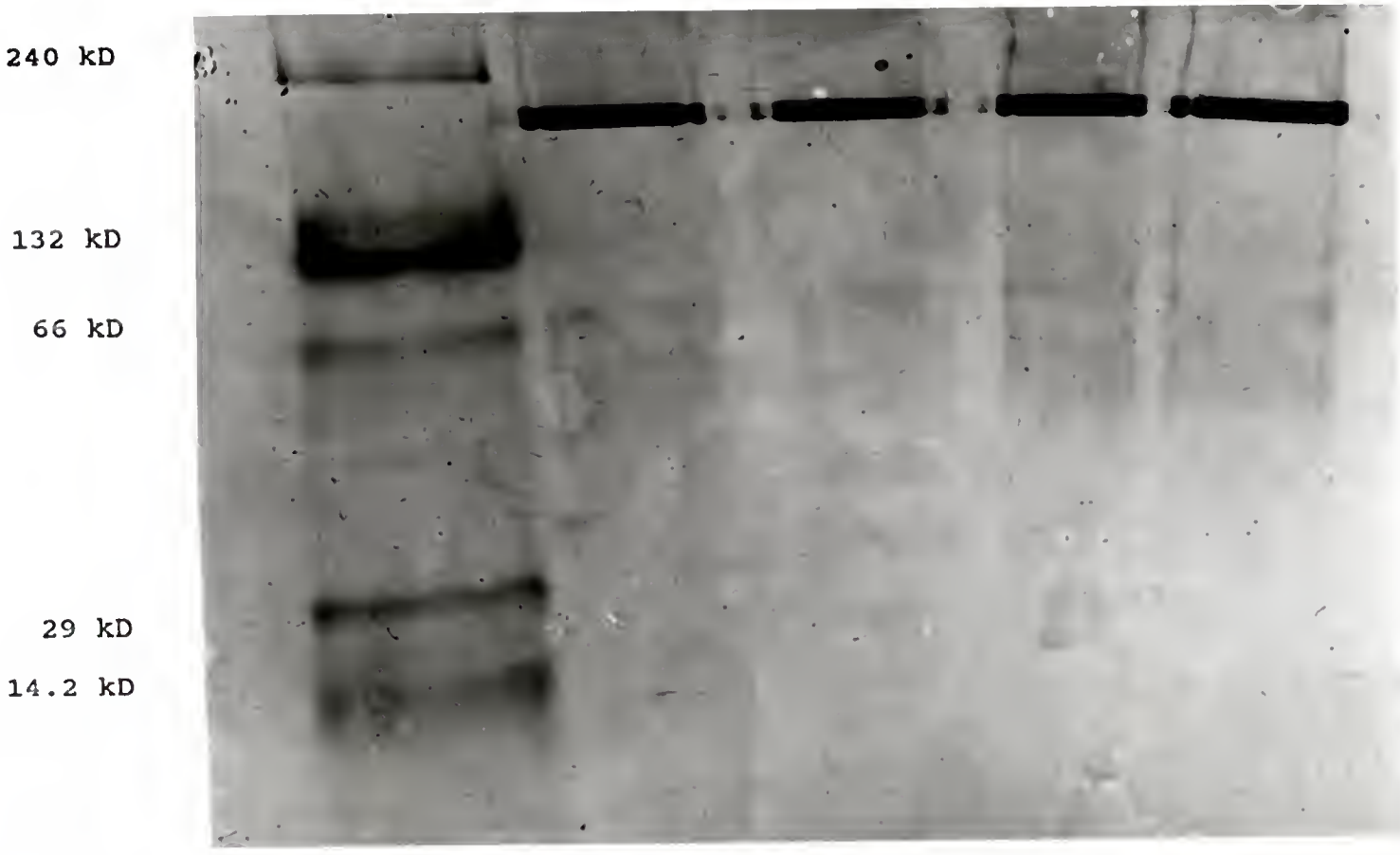

240 kD
132 kD
66 kD
29 kD
14.2 kD
Protein Standard
FSL (C)
FSL (I)
FSL (II)
FSL (III)

Table 10. Mass Balance of Protein Contents of High Pressure CO_2-treated and Nontreated PPO

		Protein Content (μg)	
		CO_2-treated	
PPO	Nontreated*	Supernatant	Pellet
Lobster	464±4@	132±7	332±5
Brown shrimp	507±5	148±3	359±6
Potato	140±2	133±4	ND#

*The total protein content based on a 1.5 mL of PPO solution was quantitated using the Bio-Rad Protein Assay kit.

@Mean value ± standard deviation

#ND, No detection

Protein patterns of CO_2-treated and nontreated lobster PPO from acrylamide gel also verified the mass balance (Figure 38). For lobster PPO, the combined protein patterns of the supernatant and the pellet portions matched those of the non-treated PPO. These results indirectly suggest that high pressure CO_2 treatment could bring about precipitation of protein molecules and thus causes PPO inactivation.

Polyacrylamide Gel Isoelectric Focusing of CO_2-treated PPO

The IEF gel patterns showed that the protein band of nontreated and CO_2 (1 atm)-treated PPO groups were at the same position (Figure 39) and the pI value was determined as 6.0. Thus, CO_2 (1 atm) treatment does not alter the electrical properties of the PPO molecule.

Untreated lobster, brown shrimp, and potato PPO only showed one protein band with an isoelectric point (pI) of 6.0 on the focused gel. Upon treatment with high pressure CO_2, the lobster, brown shrimp, and potato PPO showed several protein bands on IEF gel including one with a pI of 6.2 (Figure 40). Therefore, high pressure CO_2 treatment might cause disintegration of the PPO molecule.

Spectropolarimetric Analysis of High Pressure CO_2-treated PPO

CD spectra at the far UV range for control and high pressure CO_2-treated lobster, brown shrimp, and potato PPO are given in Figures 41, 42, and 43, respectively. The negative ellipticity between 207 and 220 nm of controls was quite different from those of CO_2-treated PPO. CO_2-treatment caused changes in the secondary structures (α-helix, β-sheet, β-turn, and random coil) (Table 11). Lobster and brown shrimp PPO showed the most

Figure 38. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE, 7.5% Gel) Profile of High Pressure (58 atm) CO_2-treated Florida Spiny Lobster (FSL) PPO; FSL (C) is Untreated PPO, While FSL (S) and FSL (P) Respectively Represent the Supernatant and Pellet Portions of High Pressure (58 atm) CO_2-treated PPO Following Centrifugation.

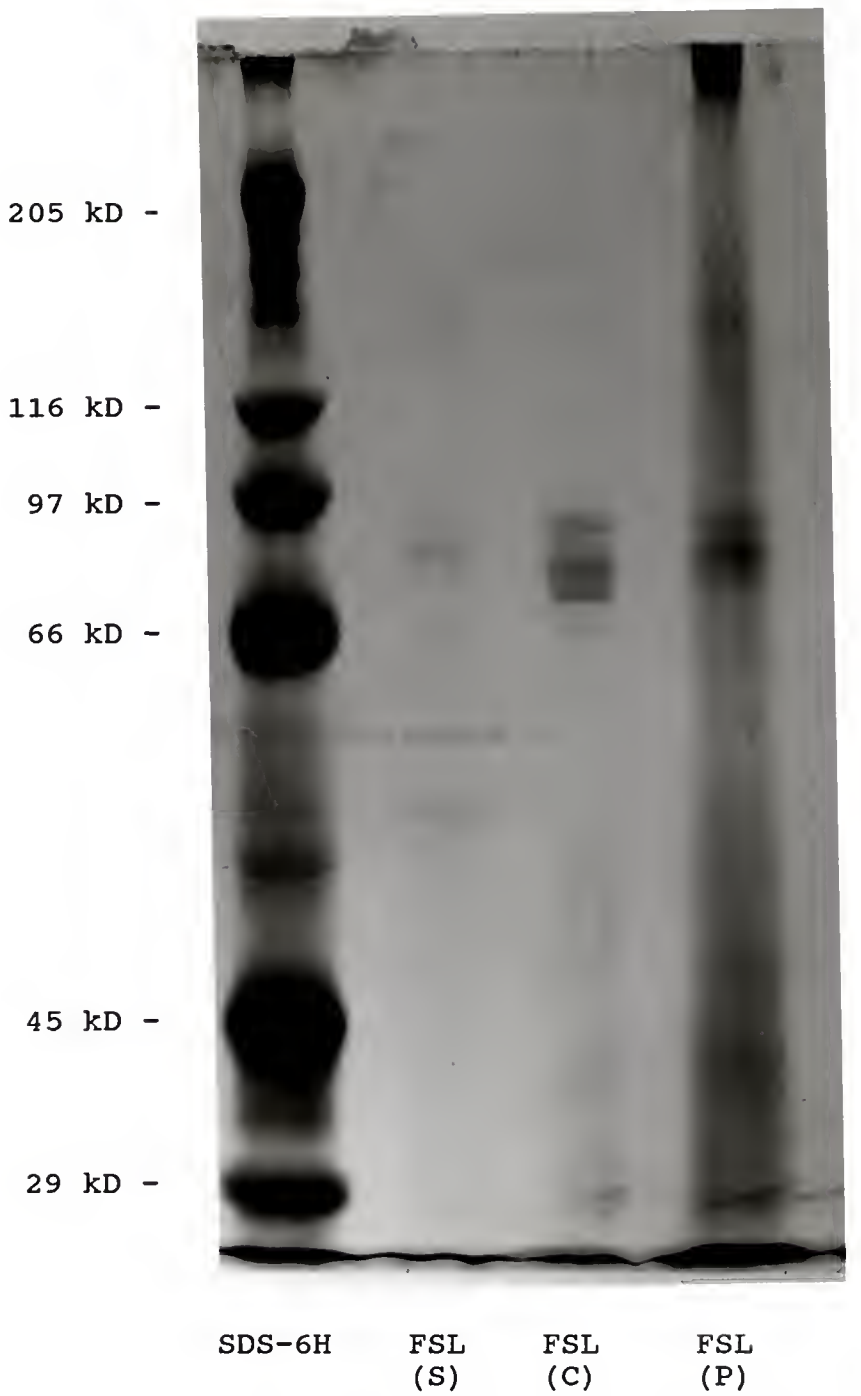

205 kD -
116 kD -
97 kD -
66 kD -
45 kD -
29 kD -
SDS-6H
FSL (S)
FSL (C)
FSL (P)

Figure 39. Polyacrylamide Gel Isoelectric Focusing (IEF, 5% Gel) Profile of Carbon Dioxide (1 atm)-treated Florida Spiny Lobster (FSL) PPO; FSL (C) Represents Non-CO_2-treated PPO, While FSL (I), FSL (II), FSL (III) Represent the PPO Heated at 33°, 38°, and 43°C, Respectively, in the Presence of CO_2. Numerical Designations Represent Isoelectric Points (pI) of the Standard Proteins.

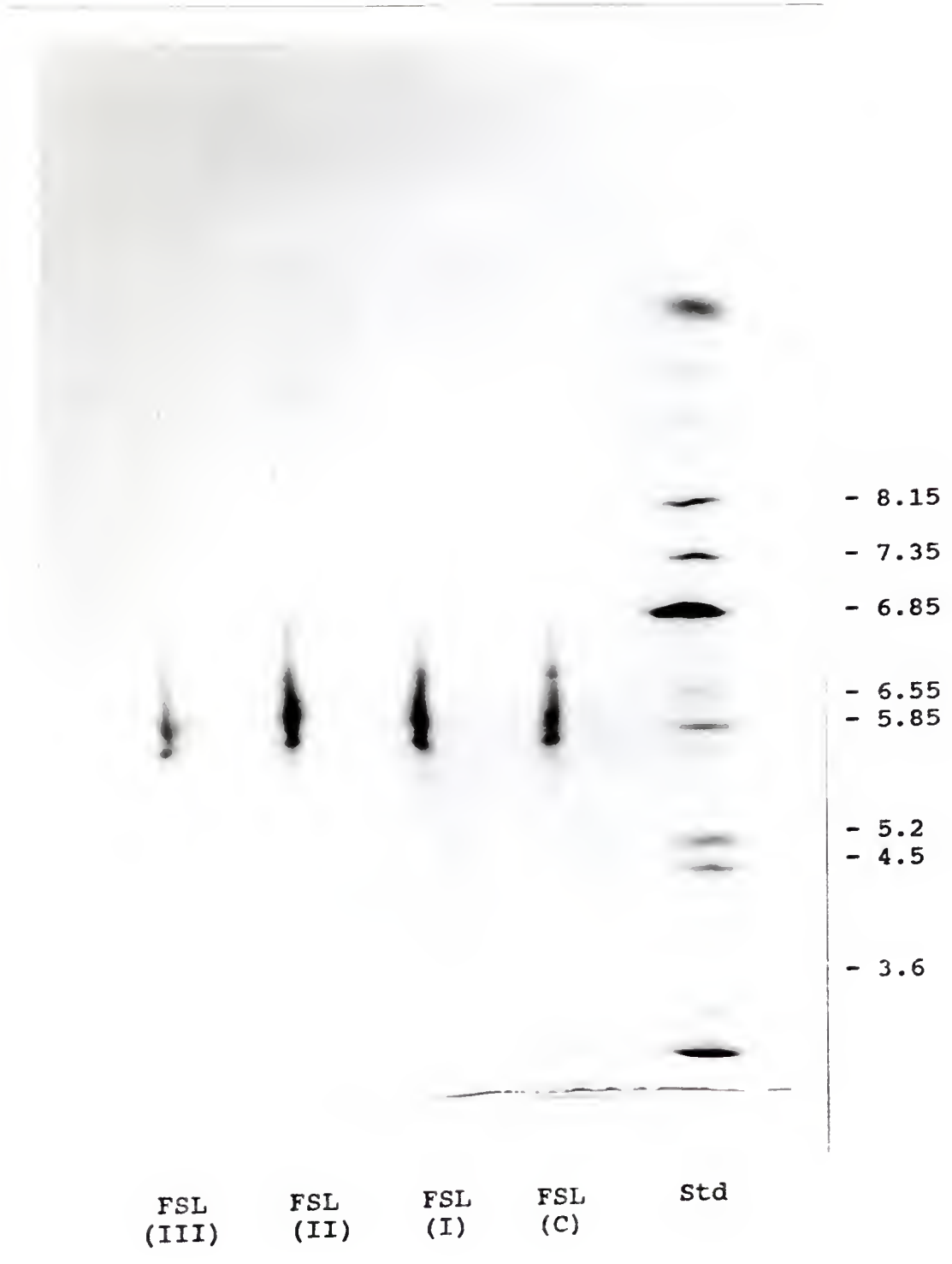

- 8.15
- 7.35
- 6.85
- 6.55
- 5.85
- 5.2
- 4.5
- 3.6
FSL (III)
FSL (II)
FSL (I)
FSL (C)
Std

Figure 40. Polyacrylamide Gel Isoelectric Focusing (IEF, 5% Gel) Profile of High Pressure (58 atm) Carbon Dioxide-treated Florida Spiny Lobster (FSL), Brown Shrimp (BS), and Potato (P) Polyphenol Oxidase (PPO). FSL (C), BS (C), and P (C) Represent Non-CO_2-treated PPO, Respectively, While FSL (T), BS (T), and P (T) Are PPO Subjected to High Pressure Treatment at 43°C. Numerical Designations Represent Isoelectric Point (pI) of the Protein Standards.

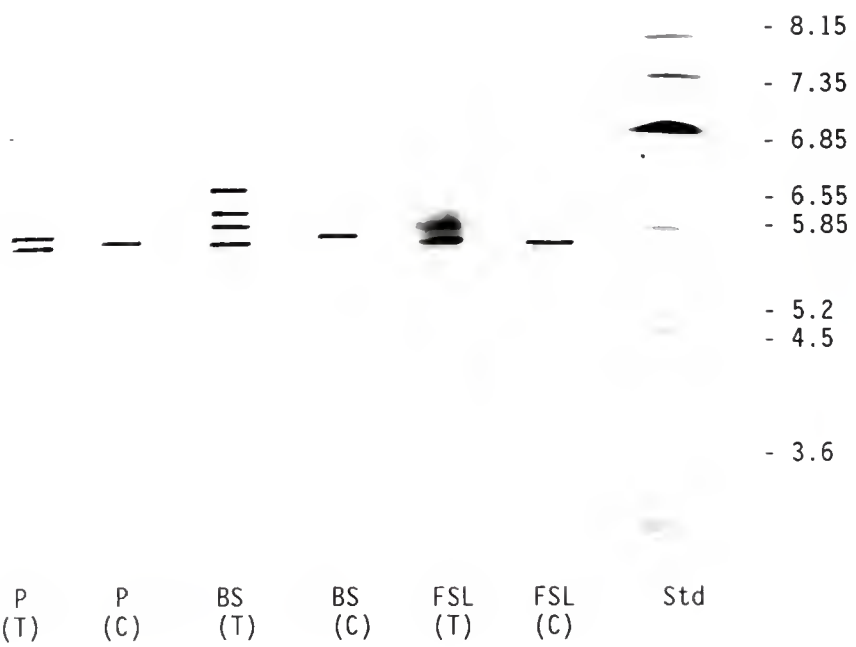

- 8.15
- 7.35
- 6.85
- 6.55
- 5.85
- 5.2
- 4.5
- 3.6
P (T)
P (C)
BS (T)
BS (C)
FSL (T)
FSL (C)
Std

Figure 41. Comparison of Far UV Circular Dichroic Spectra of Nontreated Control (----) and High Pressure (58 atm) Carbon Dioxide-treated (——) Florida Spiny Lobster PPO; Four-mL PPO (10 μg/mL) in 0.05 M Sodium Phosphate Buffer (pH 6.5) Was Analyzed at Ambient Temperature.

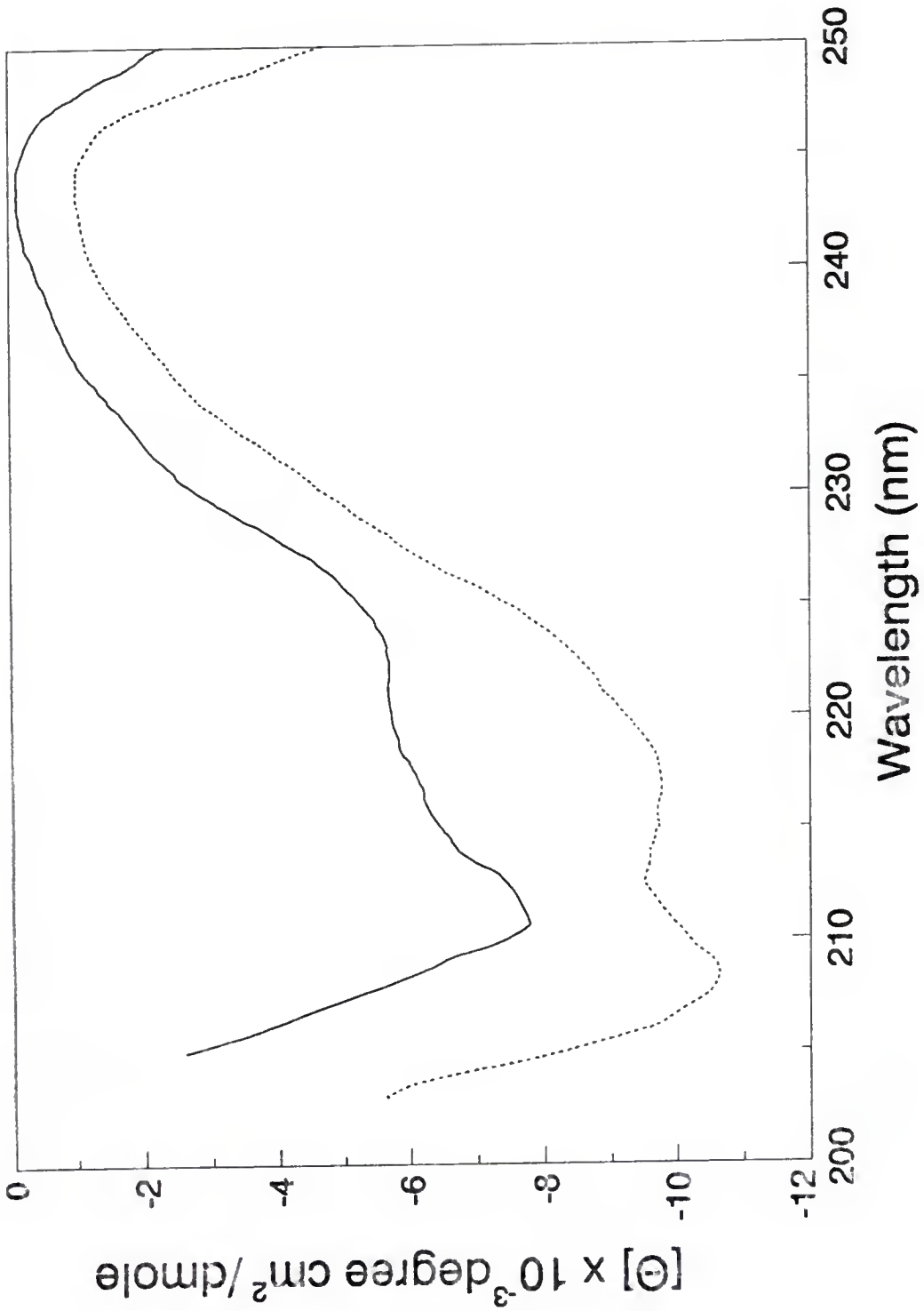

[Θ] x 10-3degree cm2/dmole
0
-2
-4
-6
-8
-10
-12
200
210
220
230
240
250
Wavelength (nm)

Figure 42. Comparison of Far UV Circular Dichroic Spectra of Nontreated Control (----) and High Pressure (58 atm) Carbon Dioxide-treated (——) Brown Shrimp PPO; Four-mL PPO (10 μg/mL) in 0.05 M Sodium Phosphate Buffer (pH 6.5) Was Analyzed at Ambient Temperature.

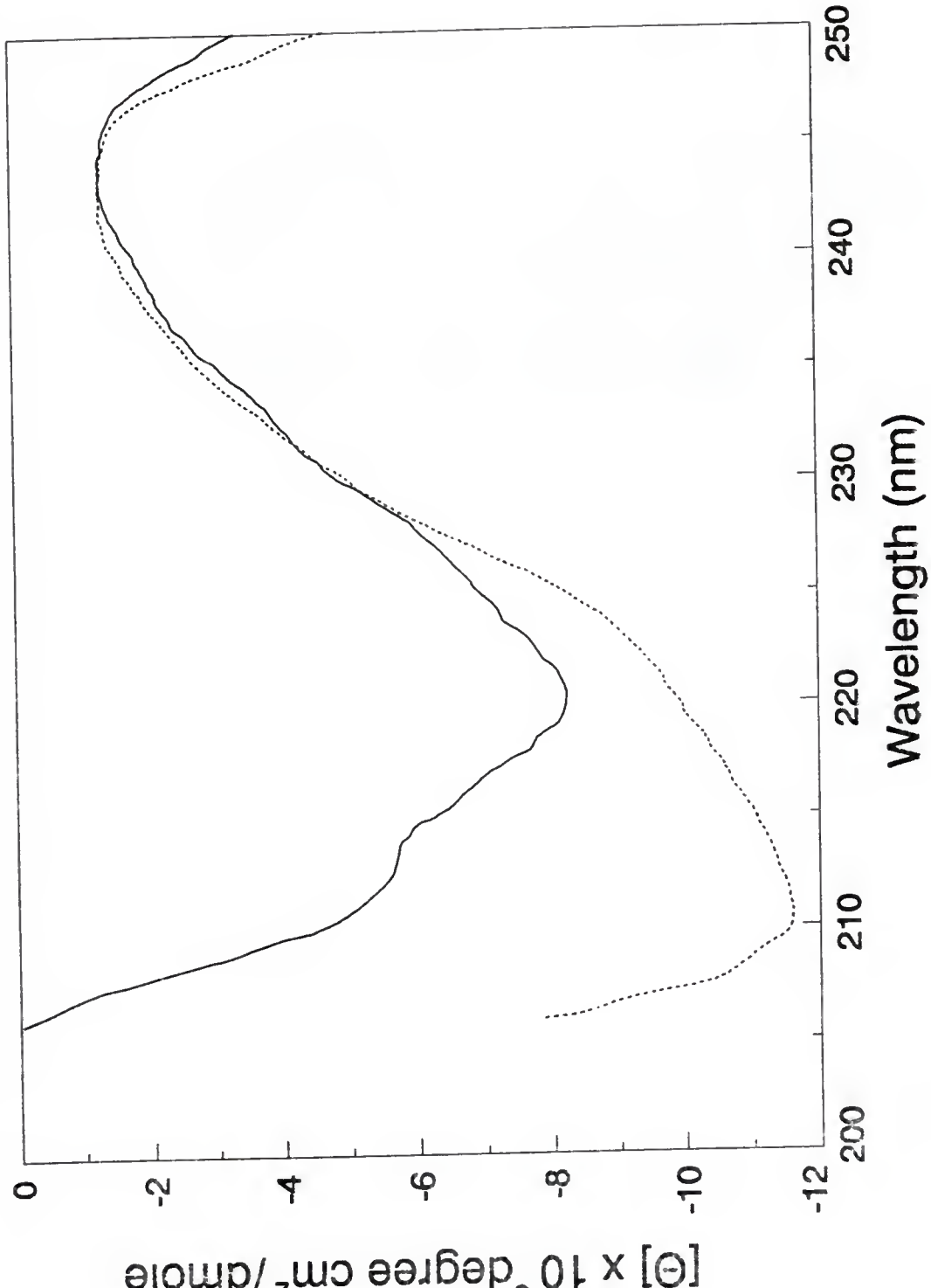

[Θ] x 10³degree cm²/dmole
0
-2
-4
-6
-8
-10
-12
200
210
220
230
240
250
Wavelength (nm)

Figure 43. Comparison of Far UV Circular Dichroic Spectra of Nontreated Control (----) and High Pressure (58 atm) Carbon Dioxide-treated (——) Potato PPO; Four-mL PPO (10 μg/mL) in 0.05 M Sodium Phosphate Buffer (pH 6.5) Was Analyzed at Ambient Temperature.

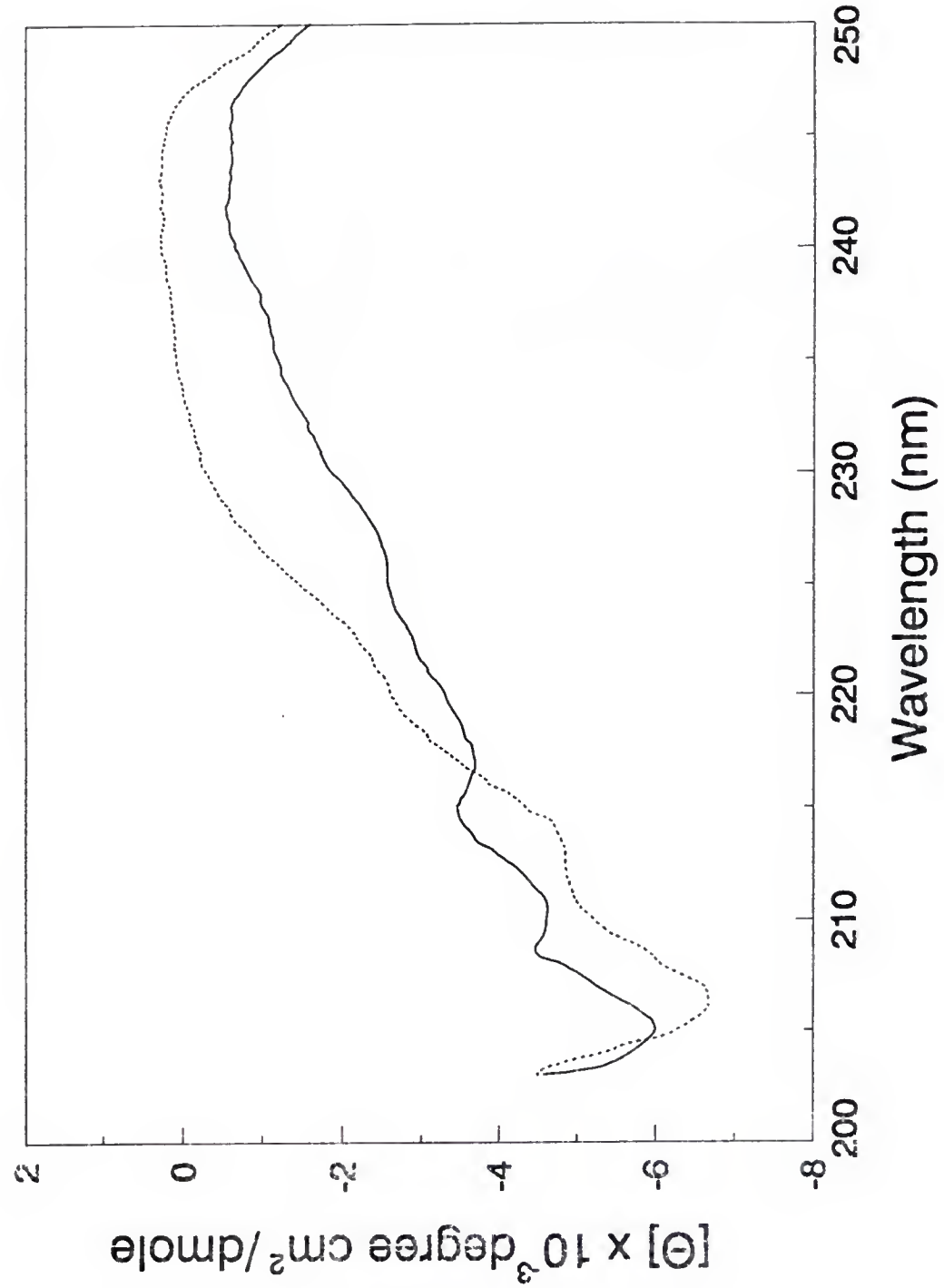

[Θ] x 10³degree cm²/dmole
2
0
-2
-4
-6
-8
200
210
220
230
240
250
Wavelength (nm)

Table 11. Secondary Structure Estimates of Nontreated Control and High Pressure CO_2-treated Florida Spiny Lobster, Brown Shrimp, and Potato Polyphenol Oxidases (PPOs) from Far UV Circular Dichroic Spectra

PPO		% of secondary structure			
		α-helix	β-sheet	β-turn	random coil
Lobster	Control	24.4	26.2	21.4	29.9
	CO_2-treated	19.7	25.9	15.2	39.3
Brown shrimp	Control	20.1	22.3	15.2	42.4
	CO_2-treated	29.6	18.9	18.2	33.3
Potato	Control	14.8	34.6	28.4	21.2
	CO_2-treated	17.8	35.9	25.9	20.4

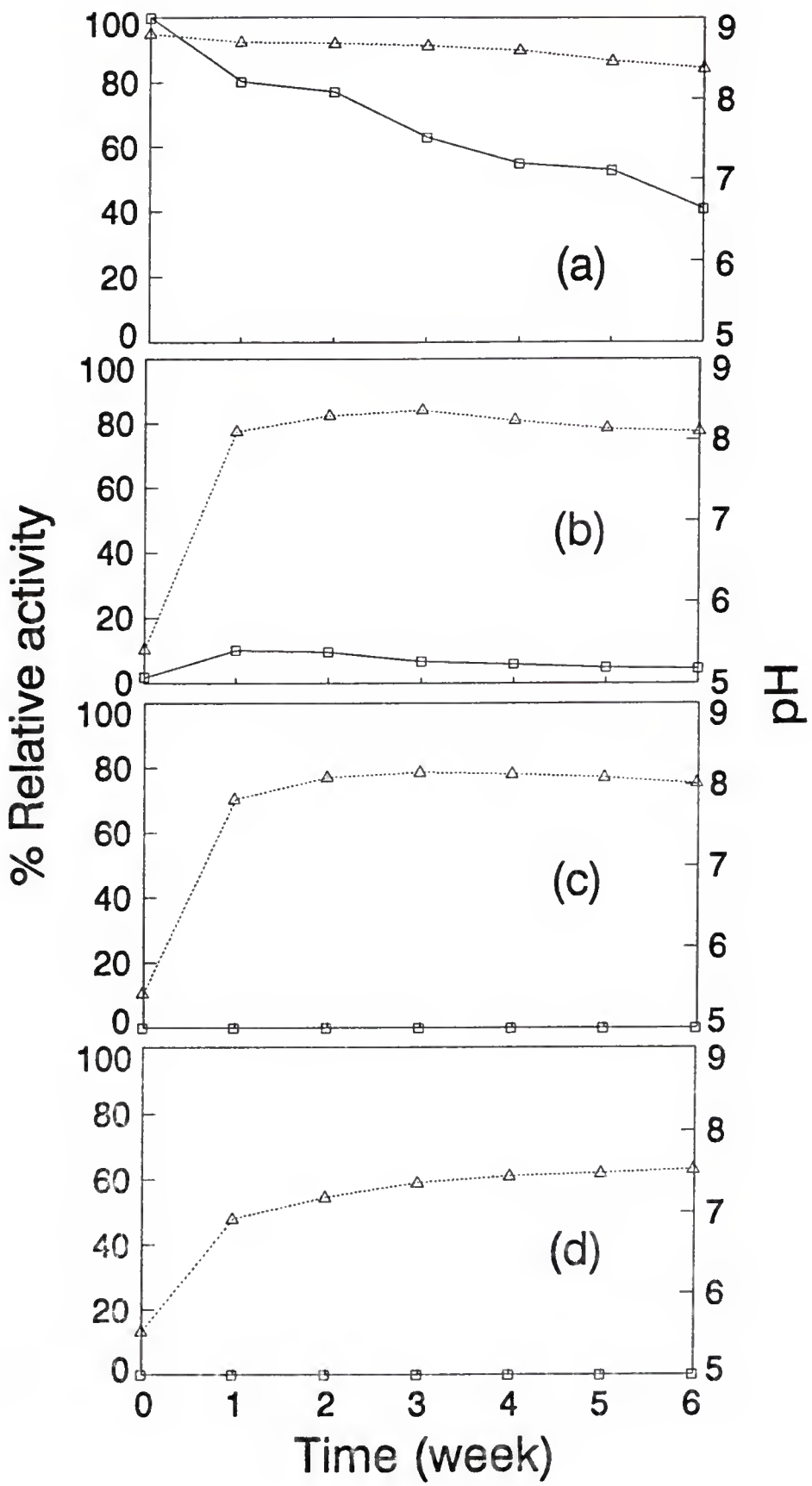
The circular dichroic spectra of PPO was scanned at the far U.V. (250 - 200 nm) range. Four-mL PPO (10 μg/mL) in 0.05 mM sodium phosphate buffer (pH 6.5) was analyzed at ambient temperature.

noticeable alterations in the composition of α-helix and random coil. In contrast, only minor alteration in secondary structures was observed in high pressure CO_2-treated potato PPO (Table 11). These results thus verified the previous finding that potato PPO was more resistant to high pressure CO_2 than lobster and brown shrimp PPO (Figures 34, 35, and 36), possibly due to its less responsiveness at altering the secondary structure. The pH change of the system and the possible bubbling effect due to CO_2, again were not completely responsible for the loss of PPO activity. This was in agreement with previous findings of Miller et al. (1981) who proposed that the pressure-induced effect from SC-CO_2 treatment could cause changes in protein backbone structure and subunit dissociation and thus inactivated the enzyme.

Restorative Ability of CO_2-treated PPO Activity

Changes in PPO activity and pH after CO_2 (1 atm) treatment and during frozen-storage are given in Figures 44a, 44b, 44c, and 44d. Non-CO_2-treated controls gradually lost PPO activity as time proceeded. Nearly 50% of the original activity (ΔA_{475nm}/min = 0.059) was lost after storage over 5 weeks (Fig. 45a). For CO_2 (1 atm)-treated-PPO, only PPO heated at 33°C was restored by 10% of its original enzyme activity (ΔA_{475nm}/min = 0.006) during the first week (Fig. 44b). After one week storage, the PPO activity decreased as storage time increased. CO_2 (1 atm)-treated PPOs heated at both 38° and 43°C showed no restoration in activities (Figures 44c and 44d). Unlike the pH change in non-CO_2-treated PPO which showed a slight decrease in pH with increased storage time (Figure 44a), those subjected to CO_2 (1 atm) treatment showed a rapid increase in pH after one

Figure 44. The Restorative Ability of Carbon Dioxide (1 atm)-treated Florida Spiny Lobster PPO Activity (□) and the Pertinent Environmental pH Changes (△) during Frozen-storage. The CO_2-treated PPO Was Heated at 33° (b), 38° (c), or 43°C (d), and the Non-CO_2-treated PPO (a) Was Run as a Control.


% Relative activity
pH
Time (week)
(a)
(b)
(c)
(d)
0
20
40
60
80
100
5
6
7
8
9
0
1
2
3
4
5
6

week of storage (Figure 44b). The pH rose from 5.4 to 8.0 for those treated-PPO at 33° and 38°C, but only to 7.2 for that heated at 43°C.

The profiles of the restorative ability of high pressure CO_2-treated lobster, brown shrimp, and potato PPOs during a 6-week frozen storage are shown in Figures 45, 46, and 47. After 6 weeks storage, the pH of high pressure CO_2-treated lobster PPO returned to 9.1 from 5.3, whereas there was no restoration of enzyme activity (Figure 45). Similarly, the pH of brown shrimp PPO rose from 4.5 to 6.50 and there was also no restoration of enzyme activity (Figure 46). For potato PPO, the pH change followed the trend similar to those observed for the previous two PPOs, climbing from 4.08 to 6.07. It was noted that 28% of the original activity (ΔA_{475nm}/min = 0.151 vs. 0.540) was restored for the CO_2-treated PPO during the first two weeks of storage (Figure 47). After this period, the PPO, however, gradually lost its activity as the storage time increased. This result was similar to the previous observation for PPO subjected to atmospheric (1 atm) CO_2 at 33°C. The treated sample restored 15% of its original enzyme activity during the first week of storage (Figure 44b).

Conclusion

Lobster PPO exposed to CO_2 (1 atm) at 33°, 38°, and 43°C showed a decline in enzyme activity with time. Inactivation kinetics study revealed that lobster PPO was more labile to the combined treatment of CO_2 and heat than to heat alone. The activities of lobster, brown shrimp, and potato PPOs followed trends similar to that of atmospheric CO_2 when these enzymes were subjected to the high pressure (58 atm) CO_2 treatment at 43°C. Results indicate PPOs were more susceptible to high pressure CO_2 than

Figure 45. The Restorative Ability of High Pressure (58 atm) Carbon Dioxide-treated Florida Spiny Lobster PPO Activity (□) and the Pertinent Environmental pH (■) Changes during Frozen-storage

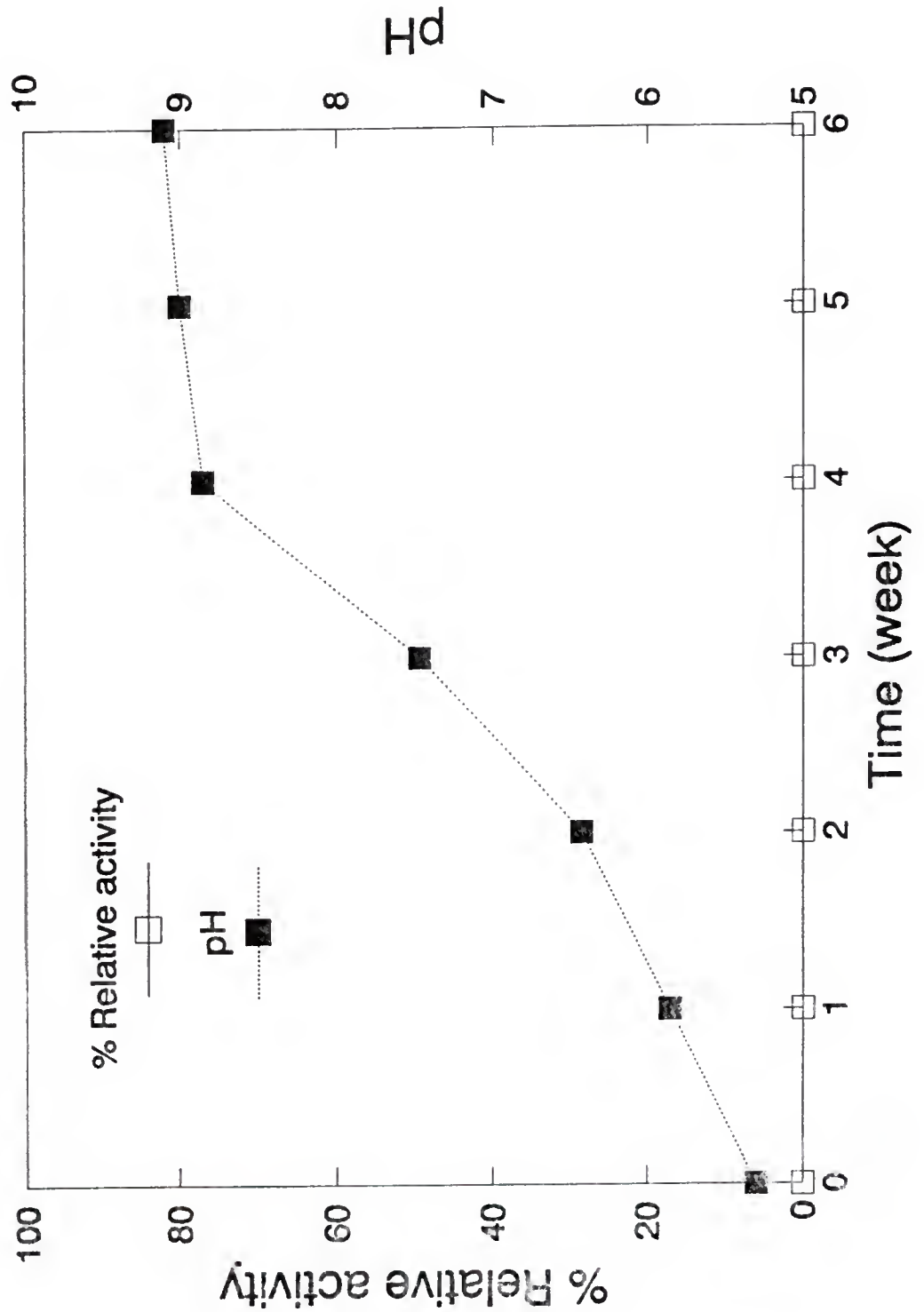

% Relative activity
pH
Time (week)
% Relative activity
pH
0
1
2
3
4
5
6
0
20
40
60
80
100
5
6
7
8
9
10

Figure 46. The Restorative Ability of High Pressure (58 atm) Carbon Dioxide-treated Brown Shrimp PPO Activity (□) and the Pertinent Environmental pH (■) Changes during Frozen-storage

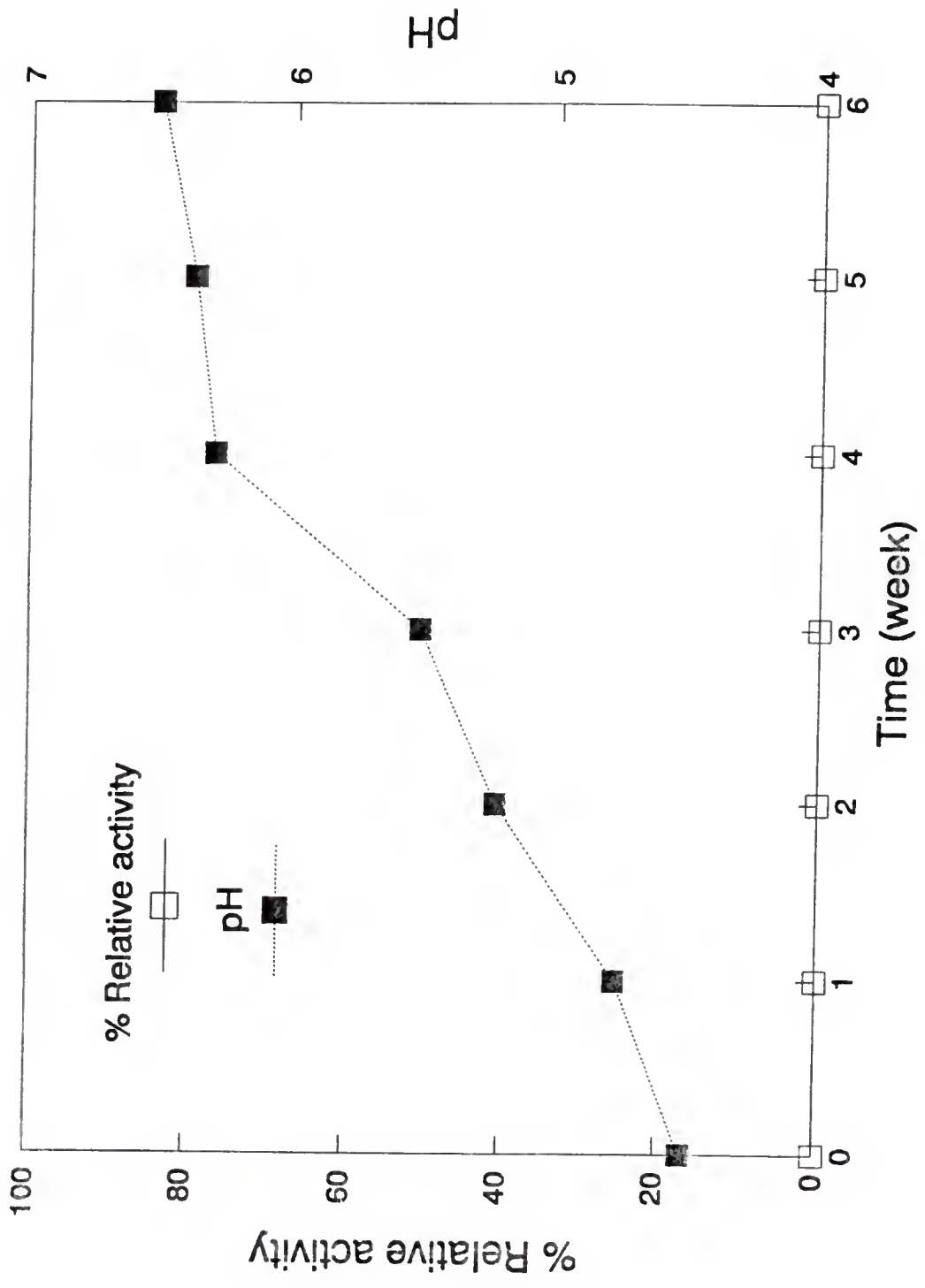
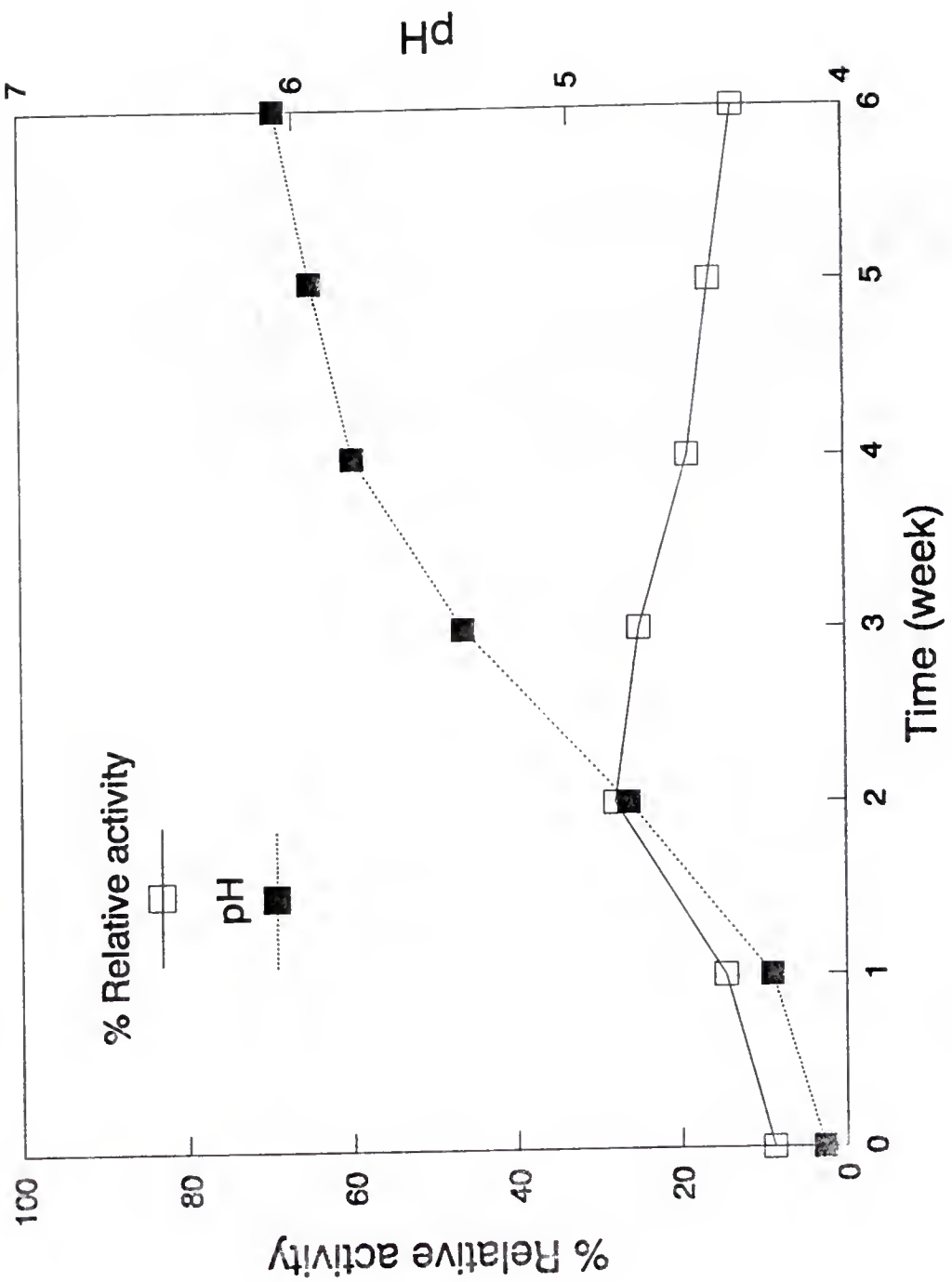

% Relative activity
pH
Time (week)
% Relative activity
pH
100
80
60
40
20
0
1
2
3
4
5
6
7
6
5
4
0
1
2
3
4
5
6

Figure 47. The Restorative Ability of High Pressure (58 atm) Carbon Dioxide-treated Potato PPO Activity (□) and the Pertinent Environmental pH (■) Changes during Frozen-storage


% Relative activity
pH
% Relative activity
pH
Time (week)
0
20
40
60
80
100
4
5
6
7
0
1
2
3
4
5
6

atmospheric CO_2; potato PPO was more resistant to high pressure CO_2 than lobster and shrimp PPOs. CO_2 (1 atm)-treated and untreated PPO did not show any differences in protein patterns and isoelectric profiles, the high pressure CO_2 treatment however affected the protein patterns and isoelectric profiles as well as secondary structures of the treated sample.

CONCLUSIONS

Polyphenol oxidase (PPO) from various sources vary with respect to their substrate specificity, kinetic properties, isoforms, molecular weights, activation energy (E_a), isoelectric point (pI) and activity and stability to pH and temperature effects. Tyrosine, phenylalanine, DOPA, and tyramine have been identified as predominant phenolic substrates of crustacean (lobster, crab, and shrimp) PPO. For mushroom and other plant PPOs, the substrate, however, varied with the sources of enzyme. Crustacean PPO had a narrow range of optimum pH between 6 to 8 compared with the broad one (4 to 7) observed for mushroom and plant PPOs. Regarding temperature optimum, crustacean PPOs had greater ranges than plant PPOs. Using immunological techniques, plant (potato and apple), mushroom, and crustacean (Florida spiny lobster, white shrimp, and brown shrimp) PPOs were shown to share similar antigenic determinants. Varied compositional secondary structures (α-helix, β-sheet, β-turn, and random coil) as revealed by spectropolarimetric analysis however existed among these different PPOs.

Kojic acid was shown to inhibit PPO activity from the various sources. It was a competitive inhibitor for the oxidation of chlorogenic acid and catechol by potato PPO and 4-methylcatechol and chlorogenic acid by apple PPO. This compound showed a mixed-type inhibition for lobster, grass prawn, and white shrimp PPO when β-3,4-dihydroxyphenylalanine (DOPA) and catechol were used as substrates, but a mixed-type and a competitive

inhibition for mushroom PPO when DOPA and L-tyrosine were used, respectively. Significant inhibitory effects with different types of inhibition mechanisms were observed with the various PPO activities when DOPA was used as substrate. In addition to mechanisms described previously, kojic acid was shown to inhibit melanosis by interfering with the uptake of oxygen required for enzymatic browning. This compound was capable of reducing *o*-quinones to *o*-diphenols; this result suggests that kojic acid could be potentially used as an inhibitor in the prevention of melanosis in plant and seafood products.

When exposed to CO_2 (1 atm) at 33°, 38°, or 43°C, lobster PPO showed a decline in enzyme activity with heating time. When these enzymes were subjected to high pressure (58 atm) CO_2 at 43°C, lobster, brown shrimp, and potato PPOs followed trends similar to that of atmospheric CO_2. Inactivation kinetics revealed lobster PPO was more labile to CO_2 and heat than heat alone. Studies showed PPOs were more susceptible to high pressure CO_2 than atmospheric CO_2; potato PPO was more resistant than lobster and shrimp PPO to the high pressure CO_2. Studies employing polyacrylamide gel electrophoresis showed there were no differences in the protein patterns and isoelectric profiles between the nontreated and CO_2 (1 atm)-treated PPO. Differences in secondary structures, protein patterns, and isoelectric profiles however were observed between the high pressure (58 atm)-treated and untreated PPO. Results from the studies suggest that low temperature (< 4°C) storage of foodstuffs that have previously been treated with CO_2 could possibly be used as a processing aid to enhance the inactivation of PPO and thus prevent enzymatic browning.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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